

AD \_\_\_\_\_  
(Leave blank)

**Award Number:**

W81XWH-04-1-0818

**TITLE:**

Castration Induced Neuroendocrine Mediated Progression of  
Prostate Cancer

**PRINCIPAL INVESTIGATOR:**

Christopher P. Evans, M.D.

**CONTRACTING ORGANIZATION:**

University of California, Davis  
Sacramento, CA 95817

**REPORT DATE:** September 2008

**TYPE OF REPORT:**

Final Addendum

**PREPARED FOR:** U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

**DISTRIBUTION STATEMENT:** (Check one)

☒ Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only;  
report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE   |                  |                                  |                                      | Form Approved<br>OMB No. 0704-0188                       |   |
|---|------------------|----------------------------------|--------------------------------------|--|---|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b> |                  |                                  |                                      |  |   |
| 1. REPORT DATE (DD-MM-YYYY)<br>30/09/08   |                  | 2. REPORT TYPE<br>Final Addendum |                                      | 3. DATES COVERED (From - To)<br>1 SEP 2007 - 31 AUG 2008 |   |
| 4. TITLE AND SUBTITLE<br><br>Castration Induced Neuroendocrine Mediated Progression of Prostate Cancer  |                  |                                  |                                      | 5a. CONTRACT NUMBER<br>-                                 |   |
|   |                  |                                  |                                      | 5b. GRANT NUMBER<br>W81XWH-04-1-0818                     |   |
|   |                  |                                  |                                      | 5c. PROGRAM ELEMENT NUMBER<br>-                          |   |
| 6. AUTHOR(S)<br><br>Christopher P. Evans, M.D.<br><br>Email: cpevans@ucdavis.edu  |                  |                                  |                                      | 5d. PROJECT NUMBER<br>-                                  |   |
|   |                  |                                  |                                      | 5e. TASK NUMBER<br>-                                     |   |
|   |                  |                                  |                                      | 5f. WORK UNIT NUMBER<br>-                                |   |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>University of California, Davis<br>Department of Urology<br>4860 Y Street, Suite 3500<br>Sacramento, CA 95817   |                  |                                  |                                      | 8. PERFORMING ORGANIZATION REPORT NUMBER<br>-            |   |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br><br>United States Army Medical<br>Research and Material Command  |                  |                                  |                                      | 10. SPONSOR/MONITOR'S ACRONYM(S)<br>-                    |   |
|   |                  |                                  |                                      | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)<br>-              |   |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for Public Release  |                  |                                  |                                      |  |   |
| 13. SUPPLEMENTARY NOTES<br>-  |                  |                                  |                                      |  |   |
| 14. ABSTRACT<br>We explored the relationship between androgen withdrawal as an event that initiates a cascade promoting the development of androgen-independent prostate cancer through neuroendocrine progression. We define the early post-castration molecular events and linked androgen-deprivation therapy to the activation of non-receptor tyrosine kinases that promote androgen-independent growth and migration. We developed an animal model to validate this hypothesis that metastasize in SCID mice. We found that deregulation through non-receptor tyrosine kinases was blocked by Src-specific inhibitor AZD0530. We found this inhibited 100% of metastasis in an animal model. The translational benefit of this study was that through a National Cancer Institute sponsored trial, the drug is being tested for hormone refractory prostate cancer.   |                  |                                  |                                      |  |   |
| 15. SUBJECT TERMS<br>Prostate Cancer, Neuroendocrine, Progression, Androgen-Independence  |                  |                                  |                                      |  |   |
| 16. SECURITY CLASSIFICATION OF:<br>U  |                  |                                  | 17. LIMITATION OF ABSTRACT<br><br>UU | 18. NUMBER OF PAGES<br><br>107                           | 19a. NAME OF RESPONSIBLE PERSON<br>Christopher P. Evans, MD |
| a. REPORT<br>U  | b. ABSTRACT<br>U | c. THIS PAGE<br>U                |                                      |  | 19b. TELEPHONE NUMBER (include area code)<br>916-734-5154   |

## Table of Contents

|                                   | Page |
|-----------------------------------|------|
| Introduction.....                 | 4    |
| Body.....                         | 4-9  |
| Key Research Accomplishments..... | 9    |
| Reportable Outcomes.....          | 10   |
| Conclusions.....                  | 14   |
| References.....                   | 14   |
| Appendices.....                   | 14   |

## DOD Final Progress Report 2008

**Introduction**

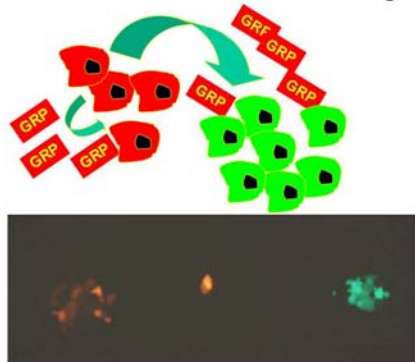
We believe that androgen withdrawal is an event that initiates a cascade promoting the development of androgen independence through NE progression. To date we know of no adjuvant therapies targeting castration initiated molecular events in clinical practice. As such, we seek to better define these early post-castration molecular events. We *hypothesize* that a small population of neuropeptide expressing AI CaP cells generated by castration can support the AI survival and growth of androgen sensitive cells in a paracrine fashion. This concept is a novel one regarding the early propagation of CaP following castration. Secondly, we *hypothesize* that neuropeptide mediated non-receptor tyrosine-kinase signaling activates androgen regulated genes both through AR and GRP dependent, and AR and GRP independent mechanisms. Demonstration of this concept establishes the rationale for neuropeptide pathway inhibition as singular and combination therapy at the time of castration.

**Body****Aim 1. To determine the paracrine effect of NE cells on androgen sensitive CaP cells.**

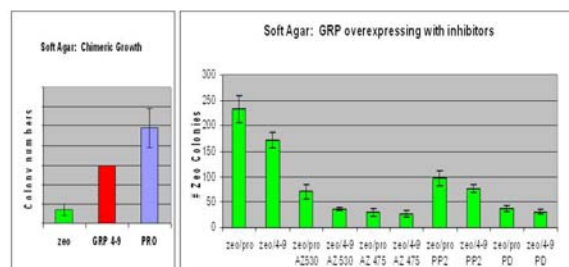
a. *Determine the in vitro ability for NE cells to support androgen sensitive CaP cell survival and growth (paracrine effect) in androgen-deprived conditions.* Work on this section was replaced by the soft agar assay as results in soft agar are more definitive.

b. *Determine the paracrine effect in soft agar tumorigenesis.* LNCaP-Zeo cells (green) do not form colonies when plated in androgen deprived soft-agar. Colony formation of LNCaP-Zeo cells (green) in soft agar assay was promoted when plated chimerically with LNCaP-GRP cells (red) (bottom left). Due to the paracrine effect of GRP expression from the GRP cells, the androgen sensitive Zeo cells formed twenty-four fold more colonies in androgen-deprived soft agar compared to when growing alone. This stimulation may be partially inhibited by a battery of Src kinase inhibitors, PP2, AZM475271, and AZD0530 (bottom right).

**Soft agar assay:  
Assess AI stimulation of AS cell growth**



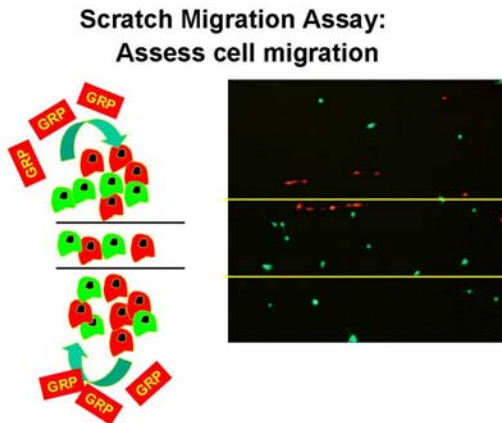
**Soft Agar: Chimeric growth of AS cells  
stimulated by AI cells**



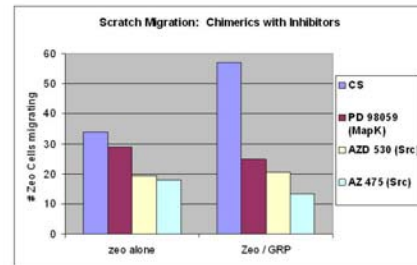
•AS cell colonies thrive when stimulated by AI cells  
•Src and MapK inhibitors attenuate this growth

c. *Determine the paracrine effect on migration in recombinant NE cells.* Stimulation of migration of LNCaP-Zeo cells by GRP cells was assessed by scratch

migration assay. This assay was conducted with the help of fluorescence tags and microscopes. LNCaP-Zeo cells do not migrate in an unstimulated environment to any significant degree. LNCaP-Zeo-GFP migrated 1.7 fold more to the scratch region when plated together with LNCaP-GRP-Red cells than alone (bottom left). MEK1 inhibitor, PD98059 and Src kinase inhibitors, AZM475271 and AZD0530 all partially inhibited this stimulated migration of LNCaP-Zeo-GFP cells (bottom right).



**Scratch Migration:**  
Chimeric stimulation of AS cells to migrate

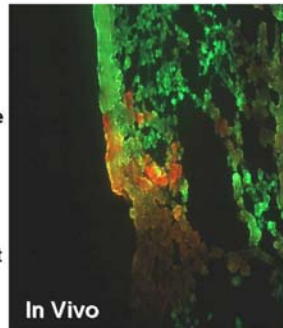


- GRP AI cells promoted migration of AS cells into lanes
- Src and MAPK inhibitors significantly reduced migration

d. *Study the paracrine effect using the in vivo xenograft model with regard to growth and metastasis.* Co-injection of LNCaP-Zeo cells with LNCaP-GRP cells in castrated SCID mice produced tumors in the prostate regions. LNCaP-Zeo cells are not normally

#### Growth of LNCaP supported in AI conditions by LNCaP-GRP

- Zeo-GFP and GRP 4-9-red cells, 2 million each, were implanted into prostates of castrated SCID mice.
- Tumor growth was palpable in 2 months.
- Frozen sections of the tumor were visualized by fluorescence microscopy.
- Cells of different origins with green or red fluorescent tags are clearly visible in tumor sections.

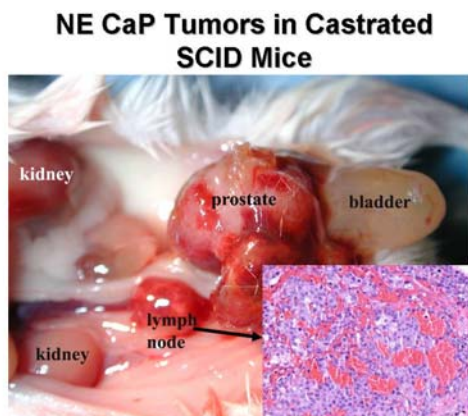


tumorigenic in the in vivo castrate environment. The Zeo cells were tagged with green fluorescence protein (GFP) and the GRP with red (Red). Frozen sections of tumor vividly showed patches of green and red colors under the fluorescent microscope. Taken together, both overexpression of GRP may stimulate growth of androgen sensitive Zeo cells both in vitro and in vivo through paracrine effect.

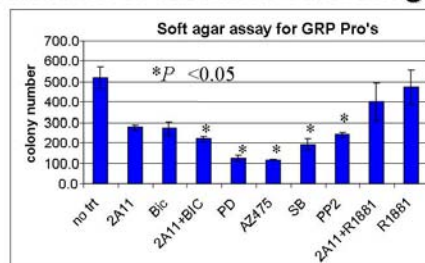
**Aim 2. To evaluate the mechanisms of AR involvement in our NE model.**

a. *Testing of inhibition of neuropeptides, signaling molecules and AR inhibitors individually and in combination on soft agar growth of GRP clones and xenograft cells.*

Tumors harvested from GRP implanted



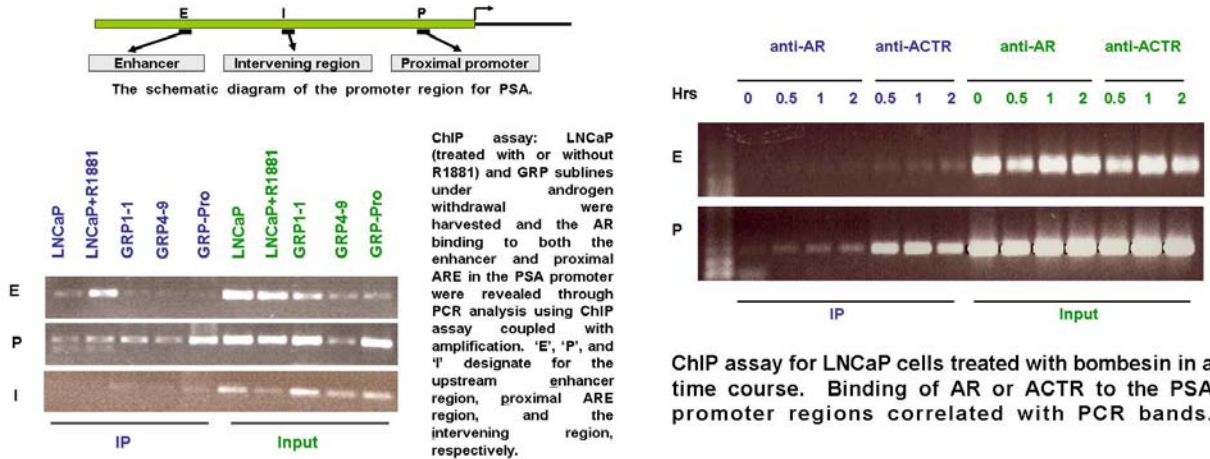
**Inhibition of Recultured Xenografts**



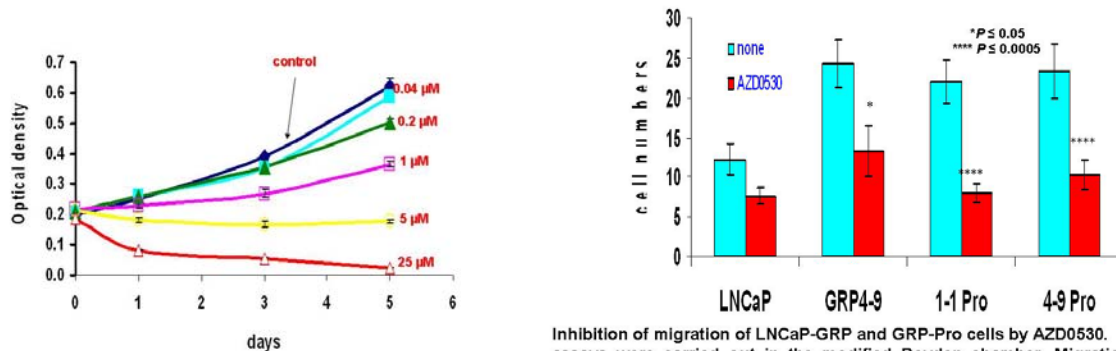
**Soft agar assay of the recultured GRP Pro's xenograft. Treatments include:** monoclonal antibody to bombesin/GRP, 2A11 (1 mg/ml), anti-androgen bicalutamide (BIC, 5  $\mu$ M), MEK1 inhibitor PD98059 (PD, 10  $\mu$ M), Src inhibitors AZM457271 (5  $\mu$ M) and PP2 (10  $\mu$ M), P38MAPK inhibitor SB 203580 (SB, 10 $\mu$ M), synthetic androgen R1881 (1  $\mu$ M).

mice were re-cultured in vitro to establish xenografts termed as GRP-Pro (derived from Prostate). The expression of human AR, PSA and GRP in tumor xenograft GRP-Pro was analyzed by RT-PCR analysis and supports the authenticity of the clones. Soft agar assay using GRP-Pro showed their aggressive nature as manifested by their androgen- and anchorage- independent growth in 2 weeks. This growth was partially inhibited by the mAb specific to bombesin, 2A11, the androgen inhibitor, bicalutamide, and in combinations (with significant difference  $p \leq 0.05$ ) supporting that the growth is dependent on both the neuropeptide GRP and AR. When synthetic androgen was added with 2A11, the colony formation ability of GRP-Pro resumed to a level similar to control. This further supports the overlapping effect of GRP and AR to the growth of GRP-Pro. Based on the tyrosine kinase display, Src kinase is present in LNCaP cells and involved in signaling via phosphorylation upon bombesin stimulation. Src kinase was constitutively active in LNCaP GRP and its xenograft GRP-Pro when cultured in androgen-free CS serum media. We thus subjected growth of LNCaP GRP-Pro to the inhibitors for Src kinases, AZM475271 from AstraZeneca and PP2. Since MEK1/2 is downstream to Src activation, we also tested the effect of PD98059. Finally, we included the MAPK P38K inhibitor SB203580 because P38 displayed activation in LNCaP cells upon androgen withdrawal. All kinase inhibitors tested decreased the growth 60-80% of control, with significant differences ( $p \leq 0.05$ ). This suggests that the androgen-independent growth of GRP-Pro involves both Src and MEK in a GRP stimulated AR-dependent manner.

The mechanisms of neuropeptide-mediated AR activation were then investigated in more detail. We performed chromatin immunoprecipitation (ChIP) assay and discovered that bombesin-stimulated AR binds preferentially to the proximal ARE site in the promoter region rather than the enhancer region bound by the androgen-stimulated AR. GRP-Pro cells constitutively expressing GRP have the AR occupied on the proximal ARE constantly. This bombesin/GRP-stimulated preferential binding of AR to the proximal site of the PSA promoter is assisted by the AR co-activator ACTR 30 min from addition of bombesin.

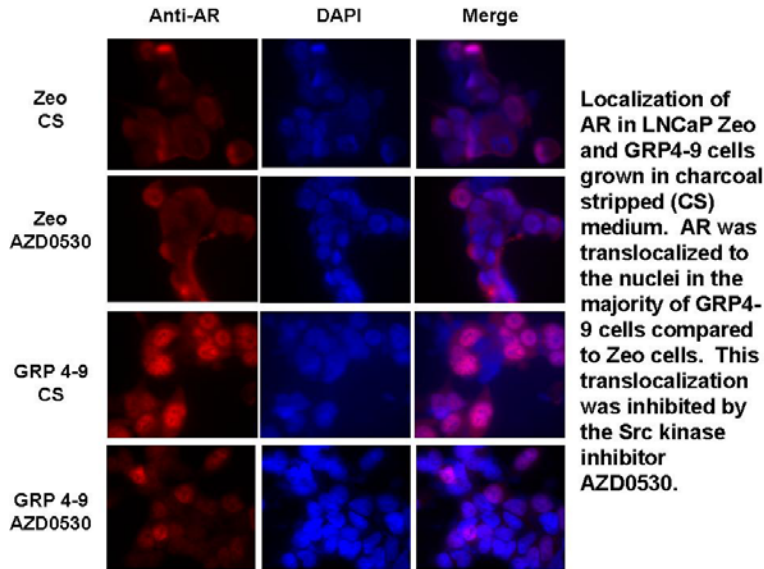


As reported last year, growth of GRP cells in soft agar may be inhibited by the specific Src inhibitor AZD0530. We performed a dose-response growth inhibition curve using GRP-Pro cells grown in CS media and treated with various doses of AZD0530. The IC<sub>50</sub> for this inhibition is slightly higher than 1  $\mu$ M. The LNCaP GRP cell lines have demonstrated promoted migratory activities than their parental cells. Src kinase inhibitor AZD0530 inhibits the migration assayed by the Boyden chamber assay to the levels similar to the basal activity in the LNCaP cells.



GRP-Pro cells were plated in CS medium with and without the Src inhibitor AZD0530 and their growth was monitored by MTT assay over 7 days. Various concentrations of AZD0530 from 0.04 to 25  $\mu$ M were added from day 0. Error bars represented standard error of means.

Inhibition of migration of LNCaP-GRP and GRP-Pro cells by AZD0530. Migration assays were carried out in the modified Boyden chamber. Migration assays were performed in a Boyden chamber with 8 mm Nucleopore membrane coated with human plasma fibronectin (50 mg/ml).  $2 \times 10^4$  LNCaP cells were placed in the upper wells, CS conditioned media with or without 500 nM AZD0530 in the lower wells, and the chamber was incubated at 37°C for 4 hours to allow cell migration. The entire field was counted under a microscope and each experiment was performed in triplicate.

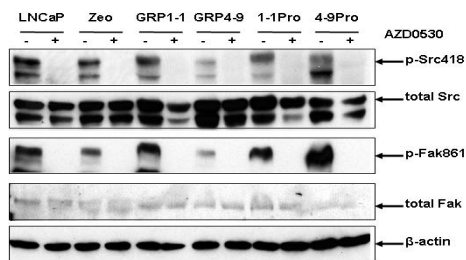


LNCaP GRP cells showed translocation of AR into the nuclei in the absence of androgen stimulation (in CS growth media) compared to the mock-transfected LNCaP Zeo cells. Addition of Src kinase inhibitor AZD0530 abolished the AR nuclear translocation as shown in the left. This result suggests that AR is activated through autocrine stimulation of GRP that is dependent of Src activation.

We surveyed the status of Src and FAK in the LNCaP and GRP subclones and found similar levels of phosphorylated Src and FAK kinases. However, when these two kinases were co-immunoprecipitated by anti-FAK antibodies, stronger phospho-Src levels were detected in GRP subclones than their mock control Zeo cells. These findings confirm our hypothesis that in the absence of AR, bombesin/GRP bind to their receptors, activate Src and FAK kinases in the complex and activate AR through phosphorylation.

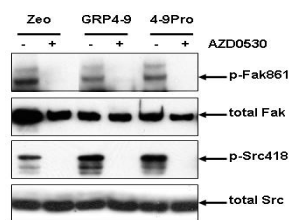
b. *Small hairpin RNA (shRNA)-based silencing of NE cells in vitro and in vivo.* We are in the process of designing the shRNA. Once we get the shRNA construct, we will start experiments in this section. We have requested the no-cost one-year extension to

#### Src and FAK status in cell lysates



Phosphorylation of Src and FAK kinases was inhibited in parental LNCaP, LNCaP-GRP and GRP-Pro cells by AZD0530 as probed by antibodies specific to Tyr(418)-Src and Tyr(861)-FAK, respectively. The ability of AZD0530 to inhibit Src-mediated phosphorylation of FAK kinase was explained by co-immunoprecipitation (co-IP) of FAK and Src kinases with anti-FAK antibody.

#### CO-IP of Src and FAK



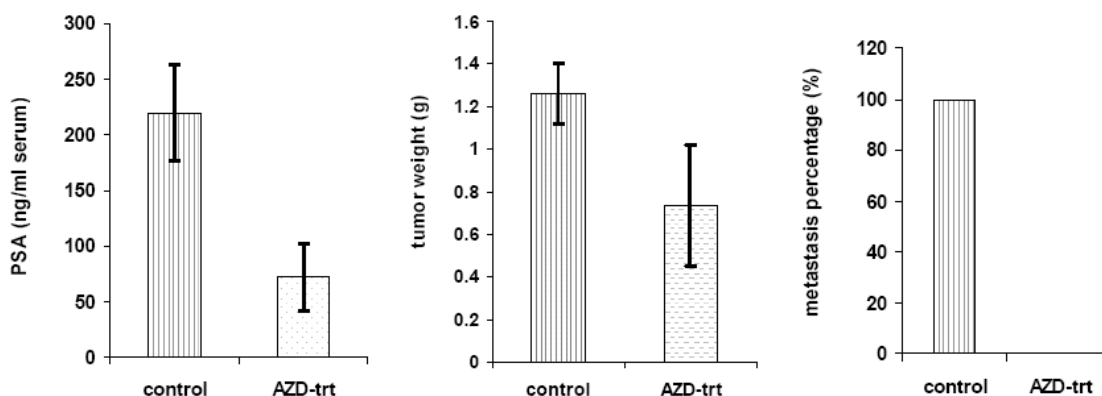
complete this and the in vivo study.

c. *Testing of inhibitory treatments on chimeric tumors in soft agar and in vivo.* We have demonstrated inhibition of paracrine migration. We are presently testing inhibition of chimeric tumor growth and metastasis in vivo.

d. *In vivo testing of inhibitory treatments at different time points.* Since we have identified Src kinase as the key player in neuropeptide-mediated AR activation, we tested the effect of Src kinase inhibitor AZD0530 in vivo with LNCaP GRP-Pro cells. After almost two months of AZD0530 administration to castrated mice injected with LNCaP GRP-Pro cells, we observed a complete inhibition of metastasis by AZD0530. Although inhibition of primary tumor growth was not significant as reported by other researchers working on various cancers, AZD0530 demonstrated potent inhibition on tumor metastasis. None of the treated animals had metastases to regional lymph nodes but both surviving control animals did. The PSA, tumor weight and metastasis data are summarized in the control and treated animals below.

**In vivo study:** Ten male SCID mice were castrated and orthotopically implanted with  $4 \times 10^6$  GRP-Pro cells into the prostate. AZD0530 (50 mg/kg) treatment was administered to seven mice (treatment group) while buffer was administered to three (control group) 16 days after surgery. The study was terminated 70 days after injection, mice from both groups were examined for primary tumor growth and metastasis. At the end of study, two remaining control mice both bore tumors and metastasis to lymph nodes, while five out of seven treated mice produced tumors but with NO metastasis.

|                  | Tumor                                  | Tumor weight (g) | Metastasis |
|------------------|--|------------------|------------|
| <b>Control</b>   | 3/3 (one died before tumor collection) | $1.04 \pm 0.34$  | 2/2        |
| <b>Treatment</b> | 5/7                                    | $0.73 \pm 0.29$  | 0/5        |



### **Other Research Accomplishments**

We have characterized the expression of the NE induced expression of src, FAK and STAT3 in all major prostate cancer cell lines. We have also validated the action of Src kinase inhibitor AZD0530 through the Src signaling pathway in two androgen-independent prostate cancer cell lines PC-3 and DU-145 by examining the status of phosphorylation of the downstream kinases and substrates. Through this study, we have identified the molecular mechanism of AZD0530. In vivo inhibitions of tumor progression by AZD0530 are also underway. These data were recently published in *Oncogene* (Aug 4, 2008, epub).

We have determined the downstream signaling cascades from NE activation and delineated the effect of a novel oral src kinase inhibitor AZD0530 at these signaling points. This data is under revised review for publication in *Cancer Research*.

### **Key Research Accomplishments**

We have demonstrated that Src kinase is the key player in neuropeptide-mediated AR activation. Together with our studies in the chimeric growth of androgen-sensitive

and androgen-insensitive cells, we are more confident with our proposed hypothesis. A paracrine effect exists for androgen insensitive CaP cells to support the survival and proliferation and migration of androgen sensitive CaP cells in a castrated environment. We have further delineated the impact of NE differentiation in prostate cancer. Most importantly, the NCI CTEP has selected AZD0530 for clinical trials, to include AICaP and based upon the above data, we were awarded this trial. Also, another Phase II AZD0530 international trial is underway in patients with prostate and breast cancer with bone metastases.

### **Reportable Outcomes**

#### **Abstract presentations 2004-2005**

1. 2004 Yang, J.C., Busby, J.E., Kung, HJ, Evans, C.P. Potent antiproliferative effects of Src kinase inhibition in a model of neuropeptide-induced androgen-independent prostate cancer. European Journal of Cancer 2(8) p.121, No. 405. (NCI/AACR/EORTC joint Molecular Therapeutics in Cancer meeting, Geneva, Switzerland).
2. 2005 Yang, J.C., Busby, J.E., Kung, HJ, Evans, C.P. Src inhibition of neuropeptide-induced androgen-independent prostate cancer. Proceedings of the American Association for Cancer Research, 46: p.748, No. 3180.
3. 2005 Evans, C.P., Busby, J.E., Kung, HJ, Yang, J.C. Androgen-sensitive prostate cancer survival and progression is supported by neuroendocrine prostate cancer cells. Proceedings of the American Association for Cancer Research, 46: p.1033, No. 4369.
4. 2005 Yang, J.C., Busby, J.E., Kung, HJ, Evans, C.P. Src kinase inhibition of neuropeptide-induced androgen-independent prostate cancer. Proceedings of the American Urological Association, 173: p.127, No. 464.

#### **Publications 2004-2005**

1. 2004 Busby, J.E., Evans, C.P. Determining variables for repeat prostate biopsy – A review. Prostate Cancer and Prostatic Diseases, 7:93-8.
2. 2004 Penson, D.F., Moul, J.W., Evans, C.P., Doyle, J.J., Gandhi, S., Stern, L, Lamerato, L. The economic burden of metastatic and prostate specific antigen progression in patients with prostate cancer: findings from a retrospective analysis of health plan data. J. Urol., 171:2250-2254.
3. 2004 Evans, C.P. Evidence-based medicine for the urologist. BJU Int., 94:1-2.
4. 2004 Busby, J. E. and Evans, C.P. Old friends, new ways: revisiting extended lymphadenectomy and neoadjuvant chemotherapy to improve outcomes. Curr Opin Urol 14:251-257.
5. 2005 Sam S. Chang, Mitchell C. Benson, Steve Campbell, Juanita Crook, Robert Dreicer, Christopher P. Evans, M. Craig Hall, Celestia Higano, W. Kevin Kelly, Oliver Sartor and Joseph A. Smith, Jr. SOCIETY OF UROLOGIC ONCOLOGY POSITION STATEMENT: REDEFINING THE MANAGEMENT OF HORMONE-REFRACTORY PROSTATE CARCINOMA. Cancer 2005;103:11-21.
6. 2005 Evans, C.P., Fleshner, N., Fitzpatrick, J. and Zlotta, A. An evidence based approach to understanding pharmacological class effect in the management of prostatic diseases. BJU Int. 2005;95:743-749.
8. 2005 Ok, J., Meyers, F. J., **Evans, C.P.** Medical and surgical palliative care of patients with urological malignancies. J. Urol. 174:1177-1182.

9. 2005 Ok, J., Cambio, A., Lara, P.N., **Evans, C.P.** Is the use of anything but MVAC justified in the evidence-based medicine era? *Curr. Opinion Urol.*, 15:312-314.

Abstract presentations 2006

1. 2006 Chang, Y-M., Bai, L., Yang, J.C., Kung, H-J., and Evans, C.P. Survey of Src activity and Src-related growth and migration in prostate cancer lines. *Proceedings of the American Association for Cancer Research*, 47: 2505.
2. 2006 Yang, J.C., Bai, L., Kung, H-J., and Evans, C.P. Androgen-sensitive prostate cancer survival and progression is supported by neuroendocrine prostate cancer cells. *Proceedings of the American Urological Association*, 175:409.
3. 2006 Evans, C.P., Bai, L., Kung, H-J., and Yang, J.C. Androgen-sensitive prostate cancer survival and progression is supported by neuroendocrine prostate cancer cells. *Urological Research Society, Salzburg Austria*.

Publications 2006

1. 2006 McGahan, J. P, Mee R., K., **Evans C.P.**, Ellison, L. Efficacy of Transhepatic Radiofrequency Ablation of Renal Cell Carcinoma. *Am. J Radiology* 2006;186:S311-S315.
2. 2006 Cambio, A. J., Evans, C.P. Management Approaches to Small Renal Tumours. *BJU Int.* 97:456-60.
3. 2006 Cambio A.J. and **Evans, C.P.** Minimising postoperative incontinence following radical prostatectomy: considerations and evidence. *Eur Urol*; 50(5):903-13; discussion 913.
4. 2006 Evans, C.P. Editorial Comment on "Penis Conserving Treatment for T1 and T2 Penile Carcinoma: Clinical Implications of a Local Recurrence. Lont, A.P. et al. *J. Urol* 2006;176:580.
5. 2006 Cambio AJ, **Evans CP.** Outcomes and quality of life issues in the pharmacological management of benign prostatic hyperplasia (BPH). *Therapeutics and Clinical Risk Management. Therapeutics and Clinical Risk Management.*, 3(1):181-196, 2007.

Abstract presentations 2007

1. 2007 Chang, Y-M., Bai, L., Yang, J.C., Kung, H-J., Evans, C.P. AZD0530 is a novel SRC kinase inhibitor with anti-proliferation and anti-migration properties in prostate cancer. *Proceedings of the American Urological Association*, 177: p.176, No. 532.
2. 2007 Yang, J.C., Chang, Y-M., Bai, L., Kung, H-J., and Evans, C.P. Inhibition of neuropeptide-mediated prostate cancer progression by specific SRC kinase inhibitor AZD0530. *Proceedings of the American Urological Association*, 177: p.221, No. 659.

Publications 2007

1. 2007 Nelson, E.C., Cambio A.J., Yang, J.C., Ok, J., Lara, P.N., **Evans CP.** Clinical Implications of Neuroendocrine Differentiation in Prostate Cancer. *Prostate Cancer and Prostatic Diseases*. 2007;10:6-14.
2. 2007 Nelson, E.C., **Evans C.P.**, Lara, P.N. Renal cell carcinoma: current status and emerging therapies. *Cancer Treat Rev.* 33:299-313.

3. 2007 Chang, Y-M., Kung, H-J, **Evans, C.P.** Non-Receptor Tyrosine Kinases in Prostate Cancer. *Neoplasia*. 2007; 9:90-100
4. 2007 Nelson, E.C., Cambio A.J. Yang, J.C., Lara, P., and **Evans, C.P.** Biologic agents as adjunctive therapy for prostate cancer: a rationale for use with androgen deprivation. *Nature Clinical Practice Urology*;4:82-94.
5. 2007 Cambio AJ, **Evans CP.** Outcomes and quality of life issues in the pharmacological management of benign prostatic hyperplasia (BPH). *Therapeutics and Clinical Risk Management*. *Therapeutics and Clinical Risk Management.*, 3(1):181-196.
6. 2007 Cambio A.J., Ellison L.M., Chamie, K., deVere White, R.W., and Evans, C.P. Cost-Benefit and outcome analysis: effect of prostate biopsy under-grading. *Urology*. 69:1152-6.
7. 2007 Nelson, E.C., **Evans, C.P.**, Mack, P.Cl, deVere White, R.W., Lara, P. Inhibition of Akt pathways in the treatment of prostate cancer. *Prostate Cancer Prostatic Diseases* 2007, *Prostate Cancer Prostatic Dis.*, 10(4):331-9.
8. 2007 Nelson EC, **Evans CP**, Pan CX, Lara PN. Prostate cancer and markers of bone metabolism: diagnostic, prognostic, and therapeutic implications. *World J Urol*. 2007 Aug;25(4):393-9. Epub 2007 Jun 12. Review.
9. 2007 **Evans CP.** Editorial Comment on: Long-Term Intravesical Adjuvant Chemotherapy Further Reduces Recurrence Rate Compared with Short-Term Intravesical Chemotherapy and Short-Term Therapy with Bacillus Calmette-Guerin (BCG) in Patients with Non-Muscle-Invasive Bladder Cancer. *Eur Urol*. epub.
10. 2007 Cambio A.J., **Evans, C.P.** and Kurzrock, E.A. A paradigm shift in the management of multicystic dysplastic kidney. *BJU Int*, in press.
11. 2007 Chee K.G., Longmate J., Quinn D.I., Chatta G., Pinski J., Twardowski P., Pan C-X, Cambio A., **Evans C.P.**, Gandara D.R., and Lara P.N. The AKT Inhibitor Perifosine in Biochemically Recurrent Prostate Cancer: A Phase II California/Pittsburgh Cancer Consortium Trial. *Clin Genitourinary Cancer*. 7:433-7.

*Abstract presentations 2008*

1. 2008 Lanfang Bai, Joy C. Yang, Phillip C. Mack, Hsing-Jien Kung and Christopher P. Evans. Targeting Src and multiple receptor tyrosine kinases (RTKs) simultaneously resulted in synergistic inhibition of proliferation and superior reduction of migration of renal cancer cells. *Proceedings of the American Association of Cancer Research, Journal of Urology*, 179(4), 34-35.
2. 2008 Bai L, Yang J, Mack P, Kung H-J, Evans C. Targeting Src and multiple receptor tyrosine kinases (RTKs) simultaneously resulted in synergistic inhibition of proliferation and superior reduction of migration of renal cancer cells. *Proceedings of the AACR Meeting Abstracts 2008*;2008:4863
3. 2008 Joy C. Yang, Lanfang Bai, Hsing-Jien Kung and Christopher P. Evans. Effect of the Specific Src Kinase Inhibitor AZD0530 on Osteolytic Lesions in Prostate Cancer. *Proceedings of the American Urological Association, The Journal of Urology* 179(4), 391.

4. Yang J, Bai L, Kung H-J, Evans C. Effect of the Src inhibitor AZD0530 on osteolytic lesions in prostate cancer. Proceedings of the AACR Meeting Abstracts 2008;2008:4984
5. 2008 Xu-Bao Shi, Lingru Xue, Joy Yang, Christopher P Evans, Ralph W deVere White, MIR-125b Induces Androgen-Independent Growth of Prostate Cancer Cells. Proceedings of the American Urological Association, The Journal of Urology 179(4), 186-187.

Publications 2008

1. 2008 Gautschi, O., Tepper, C.G., Purnell, P.R., Izumiyq, Y., **Evans, C.P.**, Green, T.P., Desprex, P.Y., Lara, P.N., Gandara, D.R., Mack, P.C., Kung, H-J. Regulation of Id1 expression by Src in cancer: implications for targeting of the bone morphogenetic protein pathway. Cancer Res 2008;68:2250-8.
2. 2008 Ramirez, M.L., Nelson, E.C., **Evans, C.P.** Beyond prostate-specific antigen: alternate serum markers. Prostate Cancer Prostatic Diseases. 2008; epub.
3. 2008 Ramirez, M.L., Nelson, E.C., de Vere White, R.W., Lara, P.N., **Evans, C.P.** Current Applications for Prostate-Specific Antigen Doubling Time. Eur. Urol. 2008; epub
4. 2008 Nelson E.C., Rodriguez R.L., Dawson K., Galvez A.F., **Evans C.P.** The interaction of genetic polymorphisms with lifestyle factors: implications for the dietary prevention of prostate cancer. Nutr. Cancer 2008;60:301-12.
5. 2008 **Evans C.P.**, Editorial comment on: Tumor Characteristics of Carriers and Noncarriers of the decode 8q24 Prostate Cancer Susceptibility Alleles. J Urol. 2008;179:2202
6. 2008 **Evans C.P.**, Editorial comment on: Barriers to the Practice of Evidence-Based Urology. J Urol. 2008;179:2350
7. 2008 Chang Y-M, Bai L, Yang J.C, Kung H-J, **Evans C.P.** Src Family Kinase Oncogenic Potential and Pathways in Prostate Cancer As Revealed By AZD0530. Oncogene, Aug 4 epub.
8. 2008 Kung H-J, **Evans C.P.** Oncogenic Activation of Androgen Receptor. Urologic Oncology, in press.
9. 2008 Joy C. Yang, Joon-ha Ok, J. Erik Busby, Alexander D. Borowsky, Hsing-Jien Kung, and Christopher P. Evans. A novel neuropeptide-autocrine model for androgen-insensitive prostate cancer: aberrant activation of androgen receptor and inhibition by AZD0530<sup>1</sup>, Revisions submitted to Cancer Research

List of Contributing Personnel

Christopher P. Evans, M.D. – Principal Investigator  
Alexander Tsodikov, Ph.D. – Biostatistician  
Joy Yang, Ph.D. – Assistant Researcher  
Yu-Ming Chang, – Graduate Student Researcher  
Lanfeng Bai – Research Specialist

## **Conclusions**

We have made headway into understanding the paracrine relationship between neuropeptide expressing, androgen-insensitive CaP cells and their ability to support the proliferation and migration of androgen sensitive CaP cells. Critically, we have identified src kinase as a molecule central to the process. We have been awarded a NIH CTEP phase II trial to study a novel, oral src kinase inhibitor AZD0530 in androgen-insensitive prostate cancer patients based upon our work. We also are involved in a Phase II trial to evaluate AZD0530 in prostate and breast cancer patients with bone metastasis.

## **References**

None

## **List of Contributing Personnel**

Christopher P. Evans, M.D. – Principal Investigator  
Alexander Tsodikov, Ph.D. – Biostatistician  
Joy Yang, Ph.D. – Assistant Researcher  
Yu-Ming Chang, – Graduate Student Researcher  
Lanfeng Bai – Research Specialist

## **Appendices** – See Attached

1. Nelson EC, Cambio AJ, Yang JC, Ok JH, Lara PN Jr, Evans CP.  
Clinical implications of neuroendocrine differentiation in prostate cancer.  
Prostate Cancer Prostatic Dis. 2007;10(1):6-14.
2. Chang YM, Kung HJ, Evans CP.  
Nonreceptor tyrosine kinases in prostate cancer.  
Neoplasia. 2007 Feb;9(2):90-100.
3. Nelson EC, Cambio AJ, Yang JC, Lara PN Jr, Evans CP.  
Biologic agents as adjunctive therapy for prostate cancer: a rationale for use with androgen deprivation.  
Nat Clin Pract Urol. 2007 Feb;4(2):82-94.
4. Nelson EC, Evans CP, Mack PC, Devere-White RW, Lara PN Jr.  
Inhibition of Akt pathways in the treatment of prostate cancer.  
Prostate Cancer Prostatic Dis. 2007 May 1
5. Nelson EC, Evans CP, Pan CX, Lara PN Jr.  
Prostate cancer and markers of bone metabolism: diagnostic, prognostic, and therapeutic implications.  
World J Urol. 2007 Aug;25(4):393-9.
6. Chang YM, Bai L, Liu S, Yang JC, Kung HJ, Evans CP.  
Src family kinase oncogenic potential and pathways in prostate cancer as revealed by AZD0530.  
Oncogene. 2008 Aug 4, epub.

7. Gautschi O, Tepper CG, Purnell PR, Izumiya Y, Evans CP, Green TP, Desprez PY, Lara PN, Gandara DR, Mack PC, Kung HJ.  
Regulation of Id1 expression by SRC: implications for targeting of the bone morphogenetic protein pathway in cancer.  
Cancer Research, 2008 Apr 1;68(7):2250-8
8. Joy C. Yang, Joon-ha Ok, J. Erik Busby, Alexander D. Borowsky, Hsing-Jien Kung, and Christopher P. Evans.  
A novel neuropeptide-autocrine model for androgen-insensitive prostate cancer: aberrant activation of androgen receptor and inhibition by AZD0530<sup>1</sup>,  
Revisions submitted to Cancer Research, 2008

## REVIEW

# Clinical implications of neuroendocrine differentiation in prostate cancer

EC Nelson, AJ Cambio, JC Yang, J-H Ok, PN Lara Jr and CP Evans

Department of Urology, Davis Medical Center, University of California at Davis, Sacramento, CA, USA

The cellular signaling pathways of the prostate play a central role in the induction, maintenance, and progression of prostate cancer (CaP). Neuroendocrine (NE) cells demonstrate attributes that suggest they are an integral part of these signaling cascades. We summarize what is known regarding NE cells in CaP focusing on NE cellular transdifferentiation. This significant event in CaP progression appears to be accelerated by androgen deprivation (AD) treatment. We examine biochemical pathways that may impact NE differentiation in a chronological manner focusing on AD therapy (ADT) as a central event in inducing androgen-independent CaP. Our analysis is limited to the common adenocarcinoma pattern of CaP and excludes small-cell and carcinoïd prostatic variants. In conclusion, we speculate on the future of treatment and research in this area. *Prostate Cancer and Prostatic Diseases* (2007) 10, 6–14. doi:10.1038/sj.pcan.4500922; published online 31 October 2006

**Keywords:** androgen-independent prostate cancer; hormone refractory; neuroendocrine cells; neuroendocrine differentiation

## Introduction

Prostate cancer (CaP) is the most common non-cutaneous malignancy in American men and is predicted to be the third leading cause of cancer deaths for 2006.<sup>1</sup> Although local therapy for CaP is relatively effective, androgen deprivation therapy (ADT) remains the mainstay of treatment for disseminated disease and is principally palliative in nature. Introduced in the 1940s,<sup>2</sup> ADT removes androgen stimulation, initially inducing apoptosis in CaP. However, the disease eventually progresses to an androgen-independent (AI) state with an associated life expectancy of only 15–20 months. Despite continuous research efforts, limited progress has been made in the treatment of advanced CaP in the last 50 years and life expectancy associated with metastatic disease has not changed significantly.<sup>3</sup> ADT, while extending length and quality of life for many patients, also induces biological changes in CaP that may promote progression to an AI state.

The role of prostatic neuroendocrine (NE) cells in this biologic process has recently become the focus of much attention. Known changes in the number, histology, and functions of NE cells during CaP progression indicate that they may play a regulatory role. The fact that the majority of NE cells may not exhibit androgen receptors (ARs) is of special interest in the androgen-deprived

patient.<sup>4,5</sup> In these patients, NE cells may allow continued CaP growth through paracrine stimulation of neoplastic epithelial cells. Indeed, mitogenic and oncogenic activity has been demonstrated for many of the factors NE cells are known to produce.

The purpose of this review is to summarize the latest developments in understanding the role of NE cells in the normal prostate, in CaP, and the effects on potential treatment modalities related to this. Data suggest that ADT may facilitate NE differentiation (NED) and thereby accelerate cellular mechanisms that contribute to the AI state. This review will be structured chronologically around this central event.

## NE histology and differentiation

The normal prostate contains a glandular epithelium within intervening fibromuscular stroma. The epithelium can be further subdivided into tall columnar cells that secrete into the lumen of the gland, and cuboidal cells forming a basal layer against the basement membrane. A third type of epithelial cell was first described by Pretl in 1944.<sup>6</sup> These cells are identified by their neurosecretory granules and expression of neuron peptide hormones such as bombesin/gastrin-releasing peptide (GRP), neurotensin (NT), serotonin, calcitonin and parathyroid hormone-related peptide (PTHrP).<sup>7,8</sup> Based on these findings, they were labeled NE cells, part of the larger amine precursor uptake and decarboxylation (APUD) lineage.

In other organs, the origin of NE cells has been shown to be endodermal stem cells,<sup>9,10</sup> and a similar model was

Correspondence: Dr CP Evans, Department of Urology, Davis Medical Center, University of California at Davis, 4860 Y Street, Suite 3500, Sacramento, CA 95817, USA.  
E-mail: christopher.evans@ucdmc.ucdavis.edu  
Received 14 September 2006; accepted 2 October 2006; published online 31 October 2006

thought to apply to prostatic NE cells. Bonkhoff *et al.*<sup>11</sup> demonstrated a possible development progression for NE cells from stem cells using immunohistochemical staining to demonstrate intermediate phenotypes. Recently, neural crest cells have re-emerged as the putative source for NE cells.<sup>12</sup> Although disagreement exists regarding the embryonic source of NE cells in the prostate, it is clear that prostatic epithelial cells are remarkably plastic and have the capability to differentiate into NE cells. As described below, the NE cells associated with CaP are phenotypically dissimilar to normal NE cells and function in different ways leading to the conclusion that they probably emerge from transdifferentiation of epithelial cells rather than malignant NE precursors.

## Normal function of NE cells

Many secreted types of NE granules are identified by immunohistochemical staining and indicate that different subsets of NE cells exist. This introduces much complexity to the question of their normal function. A general understanding may be obtained by comparisons to NE cells in other organ systems and examination of the individual products secreted by prostatic NE cells.

Prostatic NE cells are part of a larger histological genre known as the APUD system, present in many organs of the body. For example, stomach D cells and G cells produce somatostatin and gastrin, respectively, and many intestinal NE cells secrete various hormones that regulate gut function. In a similar way, it may be inferred that the factors produced by prostatic NE cells regulate prostatic growth, function, and cellular differentiation. Indeed, many of the factors shown to be produced by NE cells are known to support growth and differentiation in the prostate (Table 1). For example, bombesin/GRP receptors are members of the superfamily of heterotrimeric G-protein-coupled transmembrane-spanning receptors.<sup>13</sup> Binding of these receptors elicits calcium mobilization, thereby promoting growth and cell invasiveness through proteolytic activities in cell lines.<sup>14–17</sup>

If NE cells exert regulatory control over prostatic tissue, the question arises as to what regulates the NE cells. Although some NE cells may express AR,<sup>18</sup> many are AI as they do not contain ARs.<sup>4,5</sup> However, they do have receptors for epidermal growth factor (EGF) and ErbB2, which suggests they are controlled more by local growth factors from the prostatic stroma than systemic hormones.<sup>19</sup> The expression of the EGF receptor itself is under the control of PTHrP produced by both epithelial cells and NE cells. It has been reported that interleukin

(IL)-1 $\beta$  and IL-6 upregulate CgA expression in CaP cell lines,<sup>20</sup> and IL-6 has been shown to induce morphologic change toward an NE phenotype in epithelial cells.<sup>21</sup>

To summarize, NE cells express potent neuropeptides that mediate diverse biological processes such as cell growth, differentiation and transformation. In addition, their morphology and distribution within the prostate epithelium suggest a regulatory role similar to APUD cells in other organs of the body. In contrast to other epithelial cells, they are generally AR negative and probably rely on paracrine growth factor control.

## Role of NE cells in early CaP

The NE cells in CaP appear morphologically different than those seen in benign tissue and co-express epithelial markers such as prostate-specific antigen (PSA) and NE markers (CgA).<sup>22,23</sup> It is believed that these cells are the result of transdifferentiation of epithelial cells.<sup>24</sup> Such NED has been experimentally demonstrated in several CaP cell lines using cyclic AMP (cAMP), epinephrine, forskolin, the cytokines IL-1 and IL-6, and as will be seen later, AD conditions.<sup>20,21,25,26</sup> These changes were shown to be reversible when the substances were removed,<sup>27</sup> emphasizing the incredible degree of plasticity exhibited by prostatic epithelial cells. Based on this likely mechanism for generating malignant NE cells, some NE cells may express neuropeptide growth factors before changing morphologically and/or expressing CgA, NSE, etc. In support of this, Iwamura *et al.*<sup>28</sup> showed increased PTHrP in high-grade prostatic intraepithelial neoplasia before much NED had taken place as measured by common NE markers.

NED is very common in CaP specimens. For example, Bostwick *et al.*<sup>29</sup> reported a prevalence of 92%. Although wide ranges have been reported (30–100%), this is probably due to different sampling techniques and testing methods.<sup>30</sup> Several varieties of prostatic NED have been described. Two very rare types are small-cell carcinoma and carcinoid tumor, both of which express large numbers of malignant NE cells. The most common type is the typical adenocarcinoma with individual NE cells surrounded by small foci of epithelial CaP cells. This arrangement (Figure 1) suggests that the NE cells are producing growth factors supporting surrounding (proliferating) cells.<sup>31,32</sup>

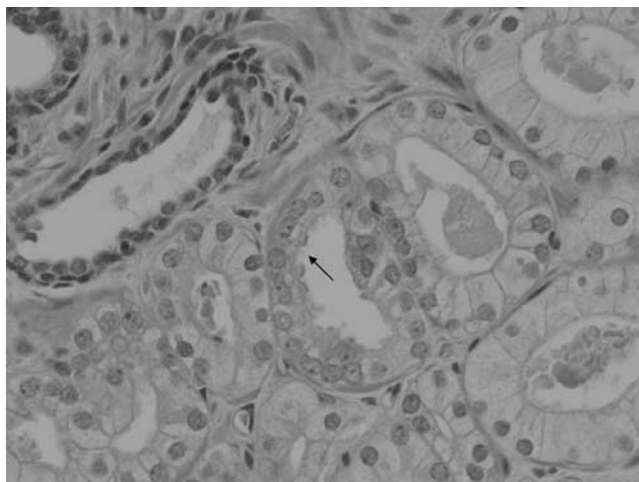
The prognostic significance of NED is controversial. Many studies before 1990 showed a worse prognosis with increasing NED, but subsequent examinations failed to find any correlation independent of tumor

**Table 1** Selected NE cellular products

| Products                             | Action in CaP   | References |
|--------------------------------------|---|------------|
| Calcitonin gene family               | Growth modulation, <i>in vitro</i> resistance to apoptosis, stimulates PTHrP release                    | 45,66      |
| GRP                                  | AI growth factor, mediates migration, <i>in vitro</i> resistance to apoptosis, activates NF- $\kappa$ B | 66,94,95   |
| Neuropeptide Y                       | Possible angiogenic effect through MAP kinase   | 96         |
| Parathyroid hormone-related protein  | Mitogenic, regulates EGF receptor, overexpressed early in CaP   | 28,77      |
| Proadrenomedullin N-terminal peptide | Angiogenesis and GRP actions through GRP receptor binding   | 97,98      |
| Serotonin                            | Mitogen, facilitates AI growth  | 24,66,99   |
| VEGF                                 | Angiogenesis, promotes growth and motility in AI manner   | 48,100     |

Abbreviations: AI, androgen-independent; CaP, prostate cancer; GRP, gastrin-releasing peptide; MAP, mitogen-activated protein; NF- $\kappa$ B, nuclear factor-kappa B; PTHrP, parathyroid hormone-related peptide; VEGF, vascular endothelial growth factor.

8 grade or androgen responsiveness.<sup>7</sup> The controversy continues with some recent studies showing an independent negative correlation between serum CgA and survival in AI CaP,<sup>33,34</sup> but others showing no prognostic correlation,<sup>35</sup> or improved outcomes with higher CgA.<sup>36</sup> These authors all agree that the serum CgA continues to be a valid marker of progression, but the complex biology of NED makes direct correlation with prognosis difficult. Cussenot *et al.*<sup>37</sup> studied CgA and NSE serum levels in CaP patients before ADT. Although most elevations of serum markers were found in AI tumors, some were not, leading them to theorize a role for NED in the progression of CaP before AD. Their



**Figure 1** NED in Gleason 7 CaP specimen (courtesy of Dr Gandour-Edwards).

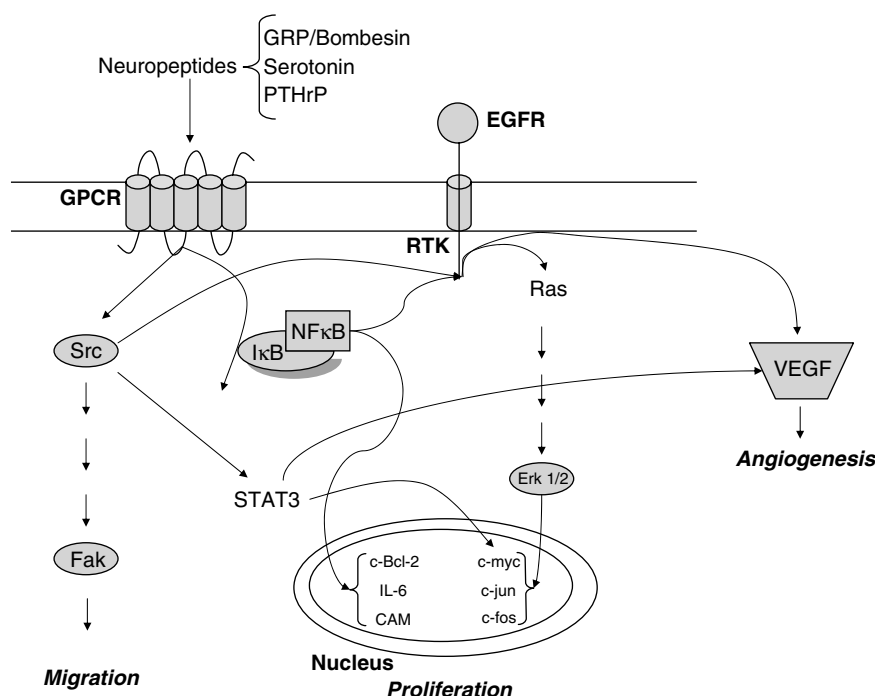
study corroborated findings by Hoosein *et al.*<sup>38</sup> that NED markers correlated more with metastasis than locally advanced disease.

The role of NE cells in the development and progression of CaP is suggested by their central role in cell signaling pathways and several of these will be briefly outlined including bombesin/GRP, serotonin, PTHrP and possible pathways involved in angiogenesis (Figure 2). A negative regulator of proliferation, somatostatin, will also be discussed.

Bombesin/GRP is a potent mitogenic neuropeptide shown to stimulate CaP growth in cell culture,<sup>39</sup> probably through its ability to induce c-fos and c-myc, thereby deregulating the cell cycle.<sup>40</sup> GRP receptors are known to be distributed throughout the human prostate and Markwalder and Reubi<sup>41</sup> showed they are over-expressed in CaP. Bombesin/GRP also causes CaP cell lines PC-3 and LNCaP to acquire greater invasive potential.<sup>42</sup>

Serotonin is produced by most NE cells and is known to be associated with malignant transformation.<sup>7,43</sup> Dizeyi *et al.*<sup>44</sup> showed several types of serotonin receptors exist in CaP tissue and cell lines. Higher grade cancer was shown to express a greater number of receptors and tissue growth was regulated by serotonin agonists and antagonists. The pathways by which serotonin acts are complex due to multiple receptor binding capabilities. It has been suggested that serotonin may be related to the potent oncoprotein ras<sup>10</sup> downstream from EGF receptors.

PTHrP is predominantly expressed in fetal tissues but is also produced by NE cells. It is overproduced by CaP tissue lines and can stimulate growth in a paracrine manner.<sup>45</sup> PSA cleaves PTHrP destroying its ability to



**Figure 2** Summary of possible pathways involving NE cellular products in the development and progression of CaP. Neuropeptides activate G-protein-coupled receptors activating Src and NF- $\kappa$ B. In addition to their direct downstream effects, they may transactivate growth factor receptors. Abbreviations: CAM, cell adhesion molecule; EGFR, epithelial growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; GPCR, G-protein-coupled receptor; NF- $\kappa$ B, nuclear factor-kappa B; RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor.

bind receptors. This has led to the suggestion that as PSA expression decreases with ADT and CaP progression, PTHrP's growth-promoting activity increases.<sup>19</sup>

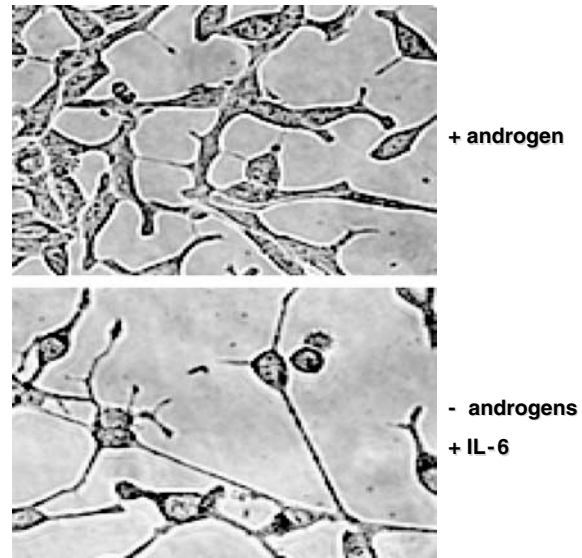
Angiogenesis is a necessary component of neoplastic growth because of increased energy requirements. NED is correlated with overall microvessel density in CaP. In addition, increased microvessel density is seen surrounding areas of NED and this effect is independent of tumor grade.<sup>46</sup> Several products of NE cells are possible mediators of this effect. Vascular endothelial growth factor (VEGF) is produced by some NE cells,<sup>47</sup> and VEGF staining NE cell density correlates with microvessel density.<sup>48</sup> Although bombesin/GRP probably does not directly stimulate angiogenesis through tyrosine kinases,<sup>49</sup> it may stimulate the nuclear factor-kappa B (NF- $\kappa$ B) angiogenesis pathway or enhance the angiogenic effects of growth factors by transactivating the EGF receptor.<sup>50–52</sup> The fact that the EGF receptor is overexpressed in CaP may enhance this effect.<sup>53</sup>

Somatostatin is the one neuropeptide that may have a restraining influence upon prostatic growth and possibly neoplastic transformation. NE cells not only produce somatostatin, they also have receptors indicating autocrine as well as paracrine function.<sup>54</sup> In CaP cells, somatostatin induces cell-cycle arrest and apoptosis,<sup>55</sup> perhaps through receptor type 3, which induces Bax.<sup>56</sup> Somatostatin may inhibit neovascularization and prostatic growth both directly and through indirect effects mediated by insulin-like growth factor (IGF)-1. Somatostatin decreases growth hormone (GH) release by the liver, which in turn decreases IGF-1 release.<sup>56</sup> Acromegalic patients have increased GH and IGF-1, and the somatostatin agonist octreotide has been shown to decrease prostate size in a cohort of these patients.<sup>57</sup>

To summarize, NE cells express potent neuropeptides that mediate diverse biological processes such as cell growth, differentiation, transformation and invasion. Although NE cells do not stain for proliferative antibodies, they may be a source of paracrine factors that support CaP growth and progression. All of these complex interactions between signal-transduction pathways are undoubtedly involved in prostatic homeostasis. This fine balance may be disturbed not only by pre-existing genetic faults but also via environmental toxins and carcinogens, diet, and the stress response, all of these acting through the microcellular hormonal milieu. Many of these microcellular environmental pathways converge through G-protein-coupled receptors via cAMP, protein kinase A and tyrosine kinases to activate mitogen-activated protein (MAP) kinases. Increasing aberrant activation of these pathways is independent of androgens, utilizing instead a complex interplay between classical growth factors and neuropeptides.

## NE cells during ADT

The current treatment of metastatic CaP consists of medical or surgical AD. The prostatic microenvironmental conditions brought on by ADT apparently play a central role in the progression of CaP to the AI state. NE cells are thought to play an important part in effecting



**Figure 3** Morphological changes in LNCaP undergoing NED. In the presence of androgen, cells show normal fusiform morphology with unbranched cellular processes. Under AD conditions, cell bodies become compact and cellular processes lengthen and demonstrate a branching morphology.

this change based on several lines of evidence from both *in vitro* and *in vivo* studies.

Jongsma *et al.*<sup>58</sup> demonstrated that PC310 cells differentiate along NE lines when they are androgen deprived. AD of LNCaP cells results in NED (Figure 3), with the addition of IL-6,<sup>21</sup> IL-8<sup>59</sup> and neuropeptides.<sup>60</sup> Human prostatic epithelial cells are similarly plastic and undergo NED in an AD mouse xenograft.<sup>61</sup> CaP patients treated with ADT demonstrate higher levels of CgA compared to androgen normal controls.<sup>62</sup> When patients undergo surgical resection, prostatic specimens showed significantly increased NED in patients treated with neoadjuvant ADT compared to surgery only.<sup>63</sup> Examination of gene expression shows higher levels of CgA mRNA in androgen-deprived CaP vs benign tissue.<sup>62</sup>

Feldman and Feldman<sup>64</sup> have described a system for classifying mechanisms of AI growth during ADT into five categories. These are by no means mutually exclusive and may all be operative. Two of their categories form a useful framework for discussing the various possible actions of NE cells in the larger tissue microenvironment, specifically their possible role in supporting AI growth of CaP epithelial cells.

The 'outlaw receptor pathway' causes androgenic effects via crosstalk between the AR and other signaling pathways. The final common pathway for these effects seems to be phosphorylation of residues on the AR causing activation of downstream effects, probably through stimulation of MAP kinase.<sup>59,65</sup> Theoretically, these signaling pathways can be activated by various biogenic amines produced by NE cells.<sup>7</sup> Jongsma *et al.*<sup>66</sup> showed that some androgen-depleted CaP cell lines can proliferate when stimulated by GRP, a neuropeptide produced by NE cells.

The gradual shift of normal to uncontrolled stimulation of proliferation may be accelerated by ADT through stimulation of greater growth factor production as shown by Culig *et al.*<sup>67</sup> for EGF. Alternatively, decreased

binding protein production may increase available growth factors. Androgens may regulate bioavailability of neuropeptides through regulation of binding proteins as has been shown for IGF/insulin-like growth factor-binding protein (IGFBP).<sup>68,69</sup>

The 'bypass pathway' includes mechanisms that do not require androgens or the AR. The inhibition of apoptosis is an important mechanism for the progression of neoplasia and several mechanisms in CaP have been studied. Bcl-2 is a gene product that blocks apoptosis and is not normally expressed by prostate epithelium.<sup>70</sup> The expression of this gene directly correlates with androgen responsiveness and has been shown to be induced upon ADT in a mouse xenograft model.<sup>71,72</sup> Epithelial cells surrounding NE cells have higher levels of Bcl-2, suggesting that the microcellular environment produced by them induces greater cell survival.<sup>73</sup> In addition, the NE cells express survivin, another antiapoptotic substance.<sup>74</sup>

Growth factors may not only promote growth through outflow receptor pathways, they may also inhibit apoptosis. Neuropeptides endothelin-1, bombesin, and growth factor IGF-1 activate the IGF-1 receptor, which phosphorylates AKT, a serine/threonine kinase. Activated AKT induces a strong antiapoptotic cellular signal.<sup>75</sup> The activity of AKT is opposed by PTEN (phosphatase and tensin homolog) and loss of PTEN is correlated with high-grade CaP.<sup>76</sup> If IGF-1 activity is increased because of the AD-mediated decrease in IGFBP, the AKT pathway would overcome the inhibition of PTEN even if it has not been lost to mutation. Activation of the AKT pathway is probably important in AI progression.<sup>77</sup>

To summarize the role of NE cells in the AI state, it has been demonstrated that the products of NE cells stimulate AI growth and increasing anaplasia. All CaP cells from cell lines and patient samples have receptors for bombesin or NT.<sup>41</sup> PC-3 cells display a growth response to NT<sup>16</sup> and invasive/motility responses to bombesin.<sup>17,42,78</sup> Elevated expression of GRP receptors are found in CaP specimens.<sup>41,79</sup> Likewise, androgen-sensitive LNCaP cells were shown to become invasive after bombesin treatment.<sup>42</sup> These and other findings suggest that NED of CaP cells may be a central link in supporting AI CaP growth under AD conditions.

## NE cells after ADT

Following ADT, NE features are an independent prognostic factor for progression of CaP.<sup>80</sup> Following neoadjuvant ADT, surgical specimens showed greater NED compared to non-treated controls.<sup>63</sup> In a retrospective study of CaP patients treated with chemotherapy, Cabrespine *et al.*<sup>35</sup> showed that CgA serum levels following ADT were independently related to treatment duration and were helpful in assessing patient response to chemotherapy.

It is unlikely that ADT always initiates NED or that this is an important feature in all CaP patients. However, if it is true that ADT of CaP tends to promote NED and supports continued progression of the tumor towards AI, then the natural question arises, can CaP associated with NED be treated?

## Treatment

Treatment of AI CaP is the focus of intense research. Only those strategies that directly impact NE cellular signaling will be discussed here. These treatments can be separated into adjunctive and salvage categories. The former combines with ADT to prevent NED from taking place, while the latter attempts to block the biochemical pathways that result from existing NED tumors.

Adjunctive treatment strategies are very limited. We are not aware of any currently used adjunctive medications intended to prevent NED. So far, the only treatments intended to do this involve variations in the method or temporal aspects of AD. Sciarra and Di Silverio<sup>81</sup> have randomized patients with biochemical progression following prostatectomy into two monotherapy groups: medical castration or antiandrogen. They showed a significantly lower CgA level in the group treated with antiandrogen, although both groups showed significant increases.

Intermittent androgen deprivation (IAD) was developed as an attempt to delay the biochemical events that lead to AI during continuous ADT. The known side effects of ADT and concern for quality of life in advanced CaP have also fueled interest. At least one study has shown that IAD may also prevent or delay NED in locally advanced disease when compared to CAD.<sup>82</sup> Metastatic disease also showed a trend toward lower serum CgA levels.

There are many treatments attempting to inhibit NED or at least block pathways NE cells use to drive CaP progression. Three known pathways that have excited interest are bombesin/GRP, serotonin and somatostatin. Antibodies against bombesin/GRP were shown to inhibit prostate cell line growth through MAP kinase pathways.<sup>83</sup> Several studies have shown *in vitro* inhibition of CaP growth using serotonin inhibitors.<sup>44,84,85</sup> Somatostatin has been used for various endocrine tumors for some time with varied success.<sup>7</sup> The actions of somatostatin in CaP are more complex and the treatment effect may be through secondary mediators such as decreasing certain growth factors from NE cells.<sup>86</sup> In addition, multiple somatostatin receptor types exist and different medications show different affinities. A recent review of the literature including seven studies using somatostatin analogues as monotherapy showed 'negative results'.<sup>87</sup> Direct growth factor antagonists of many varieties have been tested with mixed results. For example, suramin binds several growth factors and has shown moderate activity in CaP.<sup>88</sup>

Targeting downstream effectors of the pathways listed above may allow inhibition of multiple growth factors with one treatment. Src, a non-receptor tyrosine kinase activated by G-protein-coupled receptors, activates signal transducers and activator of transcription 3, which in turn activates transcription of VEGF, cyclinD1 and c-myc. Research at our institution has demonstrated the importance of Src as a central signal-transduction molecule in NED.<sup>60</sup> An NCI-sponsored phase II trial of the Src inhibitor AZD0530 as treatment for AI CaP is planned to start by early 2007.

Another indirect method of targeting growth factors is growth hormone-releasing hormone (GHRH) antagonists. These medications have recently undergone

improvements in efficacy and duration of action and have shown activity *in vitro* and in xenografts.<sup>89</sup>

Several combination therapy protocols have been used. GHRH antagonists in conjunction with bombesin/GRP antagonists showed additive interference with IGF and EGF pathways in PC-3 cell lines and xenografts.<sup>89</sup> The authors suggest this may allow future adjuvant use of these types of medication. Sciarra *et al.*<sup>90</sup> suggest that somatostatin may influence the microenvironment in which CaP cells reside, allowing other treatments to more effectively destroy the malignancy. Recognizing the direct cytotoxic effects of estrogen on CaP,<sup>91</sup> they used the somatostatin agonist lanreotide in combination with ethinyl estradiol, theorizing a synergistic effect. Fourteen of 20 stage D3 patients demonstrated extended response time and symptomatic improvement. In addition, serum CgA decreased significantly, suggesting that a decrease in NE cell number or activity may be partially responsible for their results.

Chemotherapy targets dividing cells to induce genomic damage and apoptosis. Although NE cells are typically thought to be post-mitotic,<sup>5</sup> at least one paper claims otherwise.<sup>92</sup> Modern chemotherapy regimens may be useful according to a recent report,<sup>35</sup> which demonstrated significant decreases in CgA in treated AI CaP patients.

One tremendous difficulty in developing new treatment strategies is assessing effectiveness in reaching the intended target. The focal nature of NED makes direct tissue analysis less accurate than serum markers such as CgA.<sup>93</sup> However, all currently used serum markers are expressed by non-NE cells and therefore are affected by overall prostatic tissue volume rather than only NE cell number. Measuring patient outcomes, although helpful in identifying useful treatments, gives no information on specific pathways. Development of new markers for NED is a needed area of research. Other potential methods include radiolabeled monoclonal antibody imaging studies such as somatostatin receptor scintigraphy. Non-invasive visualization of various receptors in NE tissue shows great promise in treatment assessment.

## Conclusions

In summary, AR-negative NE cells are present in normal prostatic tissue and may play a role in supporting initial neoplastic changes. ADT may induce microenvironmental changes that increase the activity of these cells. It is at least certain that they are selected for due to the lack of androgen. NE cells are capable of inducing transdifferentiation toward an NE phenotype in surrounding epithelial cells. The substances excreted by the increasing number of NE cells support the proliferation of existing CaP in an AI manner progressively increasing independence from androgen control. Greater understanding of these early post-castration molecular events will allow targeted adjunctive treatment of NED, thus decreasing the number of CaP cells that escape from hormonal control. New markers and associated imaging techniques for NED will allow molecular expression profiling, thus individualizing treatment based on the patient's unique microcellular environment.

## Acknowledgements

This work was supported in part by NIH Grant KO8 DK60748-01 and Department of Defense Grant PC040161. Mention of trade name, proprietary product or specific equipment does not constitute a guaranty of warranty by the Department of Defense, nor does it imply approval to the exclusion of other products. The views expressed herein represent those of the authors and do not necessarily represent the position of the Department of Defense.

## References

- Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C *et al*. Cancer statistics, 2006. *CA Cancer J Clin* 2006; **56**: 106–130.
- Huggins C, Hodges C. Studies on prostate cancer: I. The effect of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* 1941; **1**: 293–297.
- Eisenberger MA, Blumenstein BA, Crawford ED, Miller G, McLeod DG, Loehrer PJ *et al*. Bilateral orchiectomy with or without flutamide for metastatic prostate cancer. *N Engl J Med* 1998; **339**: 1036–1042.
- Krijnen JL, Janssen PJ, Ruizeveld de Winter JA, van Krimpen H, Schroder FH, van der Kwast TH. Do neuroendocrine cells in human prostate cancer express androgen receptor? *Histochemistry* 1993; **100**: 393–398.
- Bonkhoff H. Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. *Prostate Suppl* 1998; **8**: 18–22.
- Pretl K. Zur Frage der Endokrinie der menschlichen Vorstehdrüse. *Virchows Arch A* 1944; **312**: 392–404.
- Abrahamsson PA. Neuroendocrine cells in tumour growth of the prostate. *Endocr Relat Cancer* 1999; **6**: 503–519.
- Heasley LE. Autocrine and paracrine signaling through neuropeptide receptors in human cancer. *Oncogene* 2001; **20**: 1563–1569.
- Andrew A, Kramer B, Rawdon BB. Gut and pancreatic amine precursor uptake and decarboxylation cells are not neural crest derivatives. *Gastroenterology* 1983; **84**: 429–431.
- Tutton PJ, Barkla DH. Biogenic amines as regulators of the proliferative activity of normal and neoplastic intestinal epithelial cells [review]. *Anticancer Res* 1987; **7**: 1–12.
- Bonkhoff H, Stein U, Remberger K. Multidirectional differentiation in the normal, hyperplastic, and neoplastic human prostate: simultaneous demonstration of cell-specific epithelial markers. *Hum Pathol* 1994; **25**: 42–46.
- Aumuller G, Leonhardt M, Janssen M, Konrad L, Bjartell A, Abrahamsson PA. Neurogenic origin of human prostate endocrine cells. *Urology* 1999; **53**: 1041–1048.
- Luttrell LM, Daaka Y, Lefkowitz RJ. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 1999; **11**: 177–183.
- Aprikian AG, Han K, Chevalier S, Bazinet M, Viallet J. Bombesin specifically induces intracellular calcium mobilization via gastrin-releasing peptide receptors in human prostate cancer cells. *J Mol Endocrinol* 1996; **16**: 297–306.
- Han K, Viallet J, Chevalier S, Zheng W, Bazinet M, Aprikian AG. Characterization of intracellular calcium mobilization by bombesin-related neuropeptides in PC-3 human prostate cancer cells. *Prostate* 1997; **31**: 53–60.
- Seethalakshmi L, Mitra SP, Dobner PR, Menon M, Carraway RE. Neurotensin receptor expression in prostate cancer cell line and growth effect of NT at physiological concentrations. *Prostate* 1997; **31**: 183–192.
- Festuccia C, Guerra F, D'Ascenzo S, Giunciuglio D, Albini A, Bologna M. *In vitro* regulation of pericellular proteolysis in

- prostatic tumor cells treated with bombesin. *Int J Cancer* 1998; **75**: 418–431.
- 18 Nakada SY, di Sant'Agnese PA, Moynes RA, Hiipakka RA, Liao S, Cockett AT *et al*. The androgen receptor status of neuroendocrine cells in human benign and malignant prostatic tissue. *Cancer Res* 1993; **53**: 1967–1970.
  - 19 Iwamura M, Hellman J, Cockett AT, Lilja H, Gershagen S. Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. *Urology* 1996; **48**: 317–325.
  - 20 Diaz M, Abdul M, Hoosein N. Modulation of neuroendocrine differentiation in prostate cancer by interleukin-1 and -2. *Prostate Suppl* 1998; **8**: 32–36.
  - 21 Spiotto MT, Chung TD. STAT3 mediates IL-6-induced neuroendocrine differentiation in prostate cancer cells. *Prostate* 2000; **42**: 186–195.
  - 22 Aprikian AG, Cordon-Cardo C, Fair WR, Reuter VE. Characterization of neuroendocrine differentiation in human benign prostate and prostatic adenocarcinoma. *Cancer* 1993; **71**: 3952–3965.
  - 23 Cohen RJ, Gleason G, Haffjee Z. Prostate-specific antigen and prostate-specific acid phosphatase in neuroendocrine cells of prostate cancer. *Arch Pathol Lab Med* 1992; **116**: 65–66.
  - 24 Vashchenko N, Abrahamsson PA. Neuroendocrine differentiation in prostate cancer: implications for new treatment modalities. *Eur Urol* 2005; **47**: 147–155.
  - 25 Bang YJ, Pirnia F, Fang WG, Kang WK, Sartor O, Whitesell L *et al*. Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. *Proc Natl Acad Sci USA* 1994; **91**: 5330–5334.
  - 26 Deeble PD, Murphy DJ, Parsons SJ, Cox ME. Interleukin-6- and cyclic AMP-mediated signaling potentiates neuroendocrine differentiation of LNCaP prostate tumor cells. *Mol Cell Biol* 2001; **21**: 8471–8482.
  - 27 Cox ME, Deeble PD, Lakhani S, Parsons SJ. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. *Cancer Res* 1999; **59**: 3821–3830.
  - 28 Iwamura M, Gershagen S, Lapets O, Moynes R, Abrahamsson PA, Cockett AT *et al*. Immunohistochemical localization of parathyroid hormone-related protein in prostatic intraepithelial neoplasia. *Hum Pathol* 1995; **26**: 797–801.
  - 29 Bostwick DG, Dousa MK, Crawford BG, Wollan PC. Neuroendocrine differentiation in prostatic intraepithelial neoplasia and adenocarcinoma. *Am J Surg Pathol* 1994; **18**: 1240–1246.
  - 30 Abrahamsson PA. Neuroendocrine differentiation in prostatic carcinoma. *Prostate* 1999; **39**: 135–148.
  - 31 Bonkhoff H, Wernert N, Dhom G, Remberger K. Relation of endocrine-paracrine cells to cell proliferation in normal, hyperplastic, and neoplastic human prostate. *Prostate* 1991; **19**: 91–98.
  - 32 Bonkhoff H, Stein U, Remberger K. Endocrine-paracrine cell types in the prostate and prostatic adenocarcinoma are postmitotic cells. *Hum Pathol* 1995; **26**: 167–170.
  - 33 Berruti A, Mosca A, Tucci M, Terrone C, Torta M, Tarabuzzi R *et al*. Independent prognostic role of circulating chromogranin A in prostate cancer patients with hormone-refractory disease. *Endocr Relat Cancer* 2005; **12**: 109–117.
  - 34 Taplin ME, George DJ, Halabi S, Sanford B, Febbo PG, Hennessy KT *et al*. Prognostic significance of plasma chromogranin A levels in patients with hormone-refractory prostate cancer treated in Cancer and Leukemia Group B 9480 study. *Urology* 2005; **66**: 386–391.
  - 35 Cabrespine A, Guy L, Gachon F, Cure H, Chollet P, Bay JO. Circulating chromogranin A and hormone refractory prostate cancer chemotherapy. *J Urol* 2006; **175**: 1347–1352.
  - 36 Kim J, Palmer JL, Finn L, Hodges S, Bowes WV, Deftos L *et al*. The pattern of serum markers in patients with androgen-independent adenocarcinoma of the prostate. *Urol Oncol* 2000; **5**: 97–103.
  - 37 Cussenot O, Villette JM, Valeri A, Cariou G, Desgrandchamps F, Cortesse A *et al*. Plasma neuroendocrine markers in patients with benign prostatic hyperplasia and prostatic carcinoma. *J Urol* 1996; **155**: 1340–1343.
  - 38 Hoosein N, Abdul M, McCabe R, Gero A, Deftos LJ, Banks M *et al*. Clinical significance of elevation in neuroendocrine factors and interleukin-6 in metastatic prostate cancer. *Urol Oncol* 1995; **1**: 246–251.
  - 39 Bologna M, Festuccia C, Muzi P, Biordi L, Ciomei M. Bombesin stimulates growth of human prostatic cancer cells *in vitro*. *Cancer* 1989; **63**: 1714–1720.
  - 40 Rozengurt E, Sinnett-Smith J. Early signals underlying the induction of the c-fos and c-myc genes in quiescent fibroblasts: studies with bombesin and other growth factors. *Prog Nucleic Acid Res Mol Biol* 1988; **35**: 261–295.
  - 41 Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. *Cancer Res* 1999; **59**: 1152–1159.
  - 42 Hoosein NM, Logothetis CJ, Chung LW. Differential effects of peptide hormones bombesin, vasoactive intestinal polypeptide and somatostatin analog RC-160 on the invasive capacity of human prostatic carcinoma cells. *J Urol* 1993; **149**: 1209–1213.
  - 43 Julius D, Livelli TJ, Jessell TM, Axel R. Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation. *Science* 1989; **244**: 1057–1062.
  - 44 Dizzei N, Bjartell A, Nilsson E, Hansson J, Gadaleanu V, Cross N *et al*. Expression of serotonin receptors and role of serotonin in human prostate cancer tissue and cell lines. *Prostate* 2004; **59**: 328–336.
  - 45 Iwamura M, Wu G, Abrahamsson PA, di Sant'Agnese PA, Cockett AT, Deftos LJ. Parathyroid hormone-related protein is expressed by prostatic neuroendocrine cells. *Urology* 1994; **43**: 667–674.
  - 46 Grobholz R, Bohrer MH, Siegmund M, Junemann KP, Bleyl U, Woenckhaus M. Correlation between neovascularisation and neuroendocrine differentiation in prostatic carcinoma. *Pathol Res Pract* 2000; **196**: 277–284.
  - 47 Harper ME, Glynn-Jones E, Goddard L, Thurston VJ, Griffiths K. Vascular endothelial growth factor (VEGF) expression in prostatic tumours and its relationship to neuroendocrine cells. *Br J Cancer* 1996; **74**: 910–916.
  - 48 Borre M, Nerstrom B, Overgaard J. Association between immunohistochemical expression of vascular endothelial growth factor (VEGF), VEGF-expressing neuroendocrine-differentiated tumor cells, and outcome in prostate cancer patients subjected to watchful waiting. *Clin Cancer Res* 2000; **6**: 1882–1890.
  - 49 Busby JE, Shih SJ, Yang JC, Kung HJ, Evans CP. Angiogenesis is not mediated by prostate cancer neuropeptides. *Angiogenesis* 2003; **6**: 289–293.
  - 50 Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS *et al*. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res* 1996; **56**: 663–668.
  - 51 Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C *et al*. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 1999; **402**: 884–888.
  - 52 Levine L, Lucci III JA, Pazdrak B, Cheng JZ, Guo YS, Townsend Jr CM *et al*. Bombesin stimulates nuclear factor kappa B activation and expression of proangiogenic factors in prostate cancer cells. *Cancer Res* 2003; **63**: 3495–3502.
  - 53 Sherwood ER, Lee C. Epidermal growth factor-related peptides and the epidermal growth factor receptor in normal and malignant prostate. *World J Urol* 1995; **13**: 290–296.
  - 54 Abrahamsson PA, Anderson J, Boccon-Gibod L, Schulman C, Studer UE, Wirth M. Risks and benefits of hormonal manipulation as monotherapy or adjuvant treatment in localised prostate cancer. *Eur Urol* 2005; **48**: 900–905.
  - 55 Brevini TA, Bianchi R, Motta M. Direct inhibitory effect of somatostatin on the growth of the human prostatic cancer cell

- line LNCaP: possible mechanism of action. *J Clin Endocrinol Metab* 1993; **77**: 626–631.
- 56 Hansson J, Abrahamsson PA. Neuroendocrine pathogenesis in adenocarcinoma of the prostate. *Ann Oncol* 2001; **12** (Suppl 2): S145–S152.
- 57 Colao A, Marzullo P, Ferone D, Spiezia S, Cerbone G, Marino V *et al*. Prostatic hyperplasia: an unknown feature of acromegaly. *J Clin Endocrinol Metab* 1998; **83**: 775–779.
- 58 Jongsma J, Oomen MH, Noordzij MA, Van Weerden WM, Martens GJ, van der Kwast TH *et al*. Androgen deprivation of the PC-310 (correction of prohormone convertase-310) human prostate cancer model system induces neuroendocrine differentiation. *Cancer Res* 2000; **60**: 741–748.
- 59 Lee LF, Louie MC, Desai SJ, Yang J, Chen HW, Evans CP *et al*. Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene* 2004; **23**: 2197–2205.
- 60 Lee LF, Guan J, Qiu Y, Kung HJ. Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. *Mol Cell Biol* 2001; **21**: 8385–8397.
- 61 Huss WJ, Gregory CW, Smith GJ. Neuroendocrine cell differentiation in the CWR22 human prostate cancer xenograft: association with tumor cell proliferation prior to recurrence. *Prostate* 2004; **60**: 91–97.
- 62 Monti S, Sciarra A, Falasca P, Di Silverio F. Serum concentrations and prostatic gene expression of chromogranin A and PSA in patients affected by prostate cancer and benign prostatic hyperplasia. *J Endocrinol Invest* 2000; **23**: 53.
- 63 Ahlgren G, Pedersen K, Lundberg S, Aus G, Hugosson J, Abrahamsson PA. Regressive changes and neuroendocrine differentiation in prostate cancer after neoadjuvant hormonal treatment. *Prostate* 2000; **42**: 274–279.
- 64 Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer* 2001; **1**: 34–45.
- 65 Ueda T, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J Biol Chem* 2002; **277**: 7076–7085.
- 66 Jongsma J, Oomen MH, Noordzij MA, Romijn JC, van Der Kwast TH, Schroder FH *et al*. Androgen-independent growth is induced by neuropeptides in human prostate cancer cell lines. *Prostate* 2000; **42**: 34–44.
- 67 Culig Z, Hobisch A, Cronauer MV, Radmayr C, Hittmair A, Zhang J *et al*. Regulation of prostatic growth and function by peptide growth factors. *Prostate* 1996; **28**: 392–405.
- 68 Hampel OZ, Kattan MW, Yang G, Haidacher SJ, Saleh GY, Thompson TC *et al*. Quantitative immunohistochemical analysis of insulin-like growth factor binding protein-3 in human prostatic adenocarcinoma: a prognostic study. *J Urol* 1998; **159**: 2220–2225.
- 69 Gregory CW, Kim D, Ye P, D'Ercole AJ, Pretlow TG, Mohler JL *et al*. Androgen receptor up-regulates insulin-like growth factor binding protein-5 (IGFBP-5) expression in a human prostate cancer xenograft. *Endocrinology* 1999; **140**: 2372–2381.
- 70 McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT *et al*. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 1992; **52**: 6940–6944.
- 71 Furuya Y, Krajewski S, Epstein JI, Reed JC, Isaacs JT. Expression of bcl-2 and the progression of human and rodent prostatic cancers. *Clin Cancer Res* 1996; **2**: 389–398.
- 72 Liu AY, Corey E, Bladou F, Lange PH, Vessella RL. Prostatic cell lineage markers: emergence of BCL2+ cells of human prostate cancer xenograft LuCaP 23 following castration. *Int J Cancer* 1996; **65**: 85–89.
- 73 Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997; **3**: 917–921.
- 74 Xing N, Qian J, Bostwick D, Bergstralh E, Young CY. Neuroendocrine cells in human prostate over-express the anti-apoptosis protein survivin. *Prostate* 2001; **48**: 7–15.
- 75 Sumitomo M, Milowsky MI, Shen R, Navarro D, Dai J, Asano T *et al*. Neutral endopeptidase inhibits neuro-peptide-mediated transactivation of the insulin-like growth factor receptor-Akt cell survival pathway. *Cancer Res* 2001; **61**: 3294–3298.
- 76 McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res* 1999; **59**: 4291–4296.
- 77 Hansson J, Abrahamsson PA. Neuroendocrine differentiation in prostatic carcinoma. *Scand J Urol Nephrol Suppl* 2003; **212**: 28–36.
- 78 Aprikian AG, Tremblay L, Han K, Chevalier S. Bombesin stimulates the motility of human prostate-carcinoma cells through tyrosine phosphorylation of focal adhesion kinase and of integrin-associated proteins. *Int J Cancer* 1997; **72**: 498–504.
- 79 Bartholdi MF, Wu JM, Pu H, Troncoso P, Eden PA, Feldman RI. *In situ* hybridization for gastrin-releasing peptide receptor (GRP receptor) expression in prostatic carcinoma. *Int J Cancer* 1998; **79**: 82–90.
- 80 Krijnen JL, Bogdanowicz JF, Seldenrijk CA, Mulder PG, van der Kwast TH. The prognostic value of neuroendocrine differentiation in adenocarcinoma of the prostate in relation to progression of disease after endocrine therapy. *J Urol* 1997; **158**: 171–174.
- 81 Sciarra A, Di Silverio F. Effect of nonsteroidal antiandrogen monotherapy versus castration therapy on neuroendocrine differentiation in prostate carcinoma. *Urology* 2004; **63**: 523–527.
- 82 Sciarra A, Monti S, Gentile V, Mariotti G, Cardi A, Voria G *et al*. Variation in chromogranin A serum levels during intermittent versus continuous androgen deprivation therapy for prostate adenocarcinoma. *Prostate* 2003; **55**: 168–179.
- 83 Stangelberger A, Schally AV, Varga JL, Zarandi M, Cai RZ, Baker B *et al*. Inhibition of human androgen-independent PC-3 and DU-145 prostate cancers by antagonists of bombesin and growth hormone releasing hormone is linked to PKC, MAPK and c-jun intracellular signalling. *Eur J Cancer* 2005; **41**: 2735–2744.
- 84 Abdul M, Anezinis PE, Logothetis CJ, Hoosein NM. Growth inhibition of human prostatic carcinoma cell lines by serotonin antagonists. *Anticancer Res* 1994; **14**: 1215–1220.
- 85 Abdul M, Logothetis CJ, Hoosein NM. Growth-inhibitory effects of serotonin uptake inhibitors on human prostate carcinoma cell lines. *J Urol* 1995; **154**: 247–250.
- 86 Hejna M, Schmidinger M, Raderer M. The clinical role of somatostatin analogues as antineoplastic agents: much ado about nothing? *Ann Oncol* 2002; **13**: 653–668.
- 87 Sciarra A, Bosman C, Monti G, Gentile V, Gomez AM, Cicciariello M *et al*. Somatostatin analogues and estrogens in the treatment of androgen ablation refractory prostate adenocarcinoma. *J Urol* 2004; **172**: 1775–1783.
- 88 Kaur M, Reed E, Sartor O, Dahut W, Figg WD. Suramin's development: what did we learn? *Invest New Drugs* 2002; **20**: 209–219.
- 89 Plonowski A, Schally AV, Varga JL, Rekasi Z, Hebert F, Halmos G *et al*. Potentiation of the inhibitory effect of growth hormone-releasing hormone antagonists on PC-3 human prostate cancer by bombesin antagonists indicative of interference with both IGF and EGF pathways. *Prostate* 2000; **44**: 172–180.
- 90 Sciarra A, Cardi A, Dattilo C, Mariotti G, Di Monaco F, Di Silverio F. New perspective in the management of neuroendocrine differentiation in prostate adenocarcinoma. *Int J Clin Pract* 2006; **60**: 462–470.
- 91 Robertson CN, Roberson KM, Padilla GM, O'Brien ET, Cook JM, Kim CS *et al*. Induction of apoptosis by diethylstilbestrol in hormone-insensitive prostate cancer cells. *J Natl Cancer Inst* 1996; **88**: 908–917.

- 92 Angelsen A, Syversen U, Haugen OA, Stridsberg M, Mjølnerød OK, Waldum HL. Neuroendocrine differentiation in carcinomas of the prostate: do neuroendocrine serum markers reflect immunohistochemical findings? *Prostate* 1997; **30**: 1–6.
- 93 di Sant'Agnese PA. Neuroendocrine differentiation in carcinoma of the prostate. Diagnostic, prognostic, and therapeutic implications. *Cancer* 1992; **70**: 254–268.
- 94 Yashi M, Nukui A, Kurokawa S, Ochi M, Ishikawa S, Goto K *et al*. Elevated serum progastrin-releasing peptide (31–98) level is a predictor of short response duration after hormonal therapy in metastatic prostate cancer. *Prostate* 2003; **56**: 305–312.
- 95 Salido M, Vilches J, Lopez A, Roomans GM. Neuropeptides bombesin and calcitonin inhibit apoptosis-related elemental changes in prostate carcinoma cell lines. *Cancer* 2002; **94**: 368–377.
- 96 Zukowska-Grojec Z, Karwatowska-Prokopczuk E, Fisher TA, Ji H. Mechanisms of vascular growth-promoting effects of neuropeptide Y: role of its inducible receptors. *Regul Pept* 1998; **75–76**: 231–238.
- 97 Martinez A, Zudaire E, Portal-Nunez S, Guedez L, Libutti SK, Stetler-Stevenson WG *et al*. Proadrenomedullin NH2-terminal 20 peptide is a potent angiogenic factor, and its inhibition results in reduction of tumor growth. *Cancer Res* 2004; **64**: 6489–6494.
- 98 Ohinata K, Inui A, Asakawa A, Wada K, Wada E, Yoshikawa M. Proadrenomedullin N-terminal 20 peptide (PAMP) elevates blood glucose levels via bombesin receptor in mice. *FEBS Lett* 2000; **473**: 207–211.
- 99 Dizeyi N, Bjartell A, Hedlund P, Tasken KA, Gadaleanu V, Abrahamsson PA. Expression of serotonin receptors 2B and 4 in human prostate cancer tissue and effects of their antagonists on prostate cancer cell lines. *Eur Urol* 2005; **47**: 895–900.
- 100 Chevalier S, Defoy I, Lacoste J, Hamel L, Guy L, Begin LR *et al*. Vascular endothelial growth factor and signaling in the prostate: more than angiogenesis. *Mol Cell Endocrinol* 2002; **189**: 169–179.

# Nonreceptor Tyrosine Kinases in Prostate Cancer

Yu-Ming Chang\*, Hsing-Jien Kung<sup>\*,†,‡</sup> and Christopher P. Evans<sup>\*,‡</sup>

Departments of \*Urology and <sup>†</sup>Biological Chemistry, University of California at Davis, Sacramento, CA 95817, USA;

<sup>‡</sup>Cancer Center, University of California at Davis, Sacramento, CA 95817, USA

## Abstract

**BACKGROUND:** Carcinoma of the prostate (CaP) is the most commonly diagnosed cancer in men in the United States. Signal transduction molecules such as tyrosine kinases play important roles in CaP. Src, a nonreceptor tyrosine kinase (NRTK) and the first proto-oncogene discovered is shown to participate in processes such as cell proliferation and migration in CaP. Underscoring NRTK's and, specifically, Src's importance in cancer is the recent approval by the US Food and Drug Administration of dasatinib, the first commercial Src inhibitor for clinical use in chronic myelogenous leukemia (CML). In this review we will focus on NRTKs and their roles in the biology of CaP. **MATERIALS AND METHODS:** Publicly available literature from PubMed regarding the topic of members of NRTKs in CaP was searched and reviewed. **RESULTS:** Src, FAK, Jak1/2, and ETK are involved in processes indispensable to the biology of CaP: cell growth, migration, invasion, angiogenesis, and apoptosis. **CONCLUSIONS:** Src emerges as a common signaling and regulatory molecule in multiple biological processes in CaP. Src's relative importance in particular stages of CaP, however, required further definition. Continued investigation of NRTKs will increase our understanding of their biological function and potential role as new therapeutic targets.

*Neoplasia* (2007) 9, 90–100

**Keywords:** Nonreceptor tyrosine kinase, prostate cancer, Src, FAK, ETK.

## Introduction

Carcinoma of the prostate (CaP) is the most commonly diagnosed cancer in American men, consisting of more than 33% of all new cancer cases. Though many patients are diagnosed with CaP, it has a relatively low mortality rate when compared to other cancers. Nevertheless, it remains the third leading cause of cancer-related deaths in men in the United States, with about 27,350 estimated CaP-related deaths in 2006 in the United States [1]. Because CaP growth is facilitated by androgen exposure and because androgen withdrawal leads to apoptosis of CaP cells, the current treatment of choice for recurrent or metastatic CaP includes castration through chemical or surgical means. Nearly all patients, however, relapse with androgen-

independent (AI) disease after androgen ablation therapy. Ultimately, the uncontrolled growth of AI metastatic tumors leads to patient mortality.

Tyrosine kinases (TKs) are signaling molecules well known for their roles in human diseases such as diabetes and cancer. Indeed, v-Src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src), a nonreceptor tyrosine kinase (NRTK), was the first proto-oncogene discovered. More than a quarter of a century has passed since the discovery of Src, and the studies on TKs are coming to fruition with the development and use of tyrosine kinase-based target-specific therapy such as Gleevec, Iressa, and Herceptin for therapy against chronic myelogenous leukemia (CML), lung cancer, and breast cancer, respectively. Dasatinib, a dual Src/v-Abl Abelson murine leukemia viral oncogene homolog (Abl) inhibitor with anti-migratory activity in prostate cancer cells in culture was recently approved by the US Food and Drug Administration for use in patients with CML [2]. Further underscoring the importance of NRTKs, AZD0530 is another dual Src/Abl inhibitor that is currently in multicenter phase II clinical trials for multiple types

Abbreviations: Abl, v-Abl Abelson murine leukemia viral oncogene homolog; AI, androgen-independent; Akt, v-akt murine thymoma viral oncogene homolog 1; AR, androgen receptor; ARG, Abelson-related gene; Bcr, breakpoint cluster region; Brk/PTK6, breast tumor kinase/protein tyrosine kinase 6; BPH, benign prostatic hypertrophy; BRCA1, breast cancer susceptibility gene 1; CaP, carcinoma of the prostate; CML, chronic myelogenous leukemia; CRKII, v-crk avian sarcoma virus CT10 oncogene homolog; CSK, C-terminal Src kinase; DOC-2/DAB2, differentially expressed in ovarian cancer-2/disabled-2; EGF, epidermal growth factor; ER, estrogen receptor; ERK1/2, extracellular signal-regulated kinase 1/2; ET1, endothelin; ETK/BMX, endothelial/epithelial tyrosine kinase/bone marrow X kinase; FAK, focal adhesion kinase; FeR, FpS/FeS-related tyrosine kinase; FeS/FpS, feline sarcoma oncogene/fujinami avian sarcoma viral oncogene homolog; FGR, Gardner-Rasheed feline sarcoma viral (v-FGR) oncogene homolog; Fyn, Fyn oncogene related to Src, FGR, Yes; HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; IGF-1, insulin-like growth factor 1; IL, interleukin; Jak, Janus kinase; KAI1/CD82, Kangai 1/cluster designation 82; Lck, lymphocyte-specific protein tyrosine kinase; Lyn, v-Yes-1 Yamaguchi sarcoma viral-related oncogene homolog; LPA, lysophosphatidic acid; Met, met proto-oncogene (hepatocyte growth factor receptor); MMP, matrix metalloproteinase; NEP, neutral endopeptidase; NRTK, nonreceptor tyrosine kinase; p130CAS, p130 CRK-associated substrate; PAK1, p21-associated kinase 1; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog; PYK2/CAK $\beta$ , proline-rich tyrosine kinase 2/cell adhesion kinase  $\beta$ ; Raf, v-raf-1 murine leukemia viral oncogene homolog 1; Ras, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; SH, Src homology; Src, v-Src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog; STAT, signal and transducer of transcription; SYK, spleen tyrosine kinase; Tec, Tec protein kinase; TGF, tumor growth factor; TIMP, tissue inhibitor of metalloproteinase; TKIP, tyrosine kinase inhibitor peptide; TnK, tyrosine kinase nonreceptor; Tyk2, tyrosine kinase 2; VEGF, vascular endothelial growth factor; Yes, v-Yes-1 Yamaguchi sarcoma viral oncogene homolog 1

Address all correspondence to: Christopher P. Evans, Department of Urology, University of California at Davis, Suite 3500, 4860 Y Street, Sacramento, CA 95817.

E-mail: cpevans@ucdavis.edu

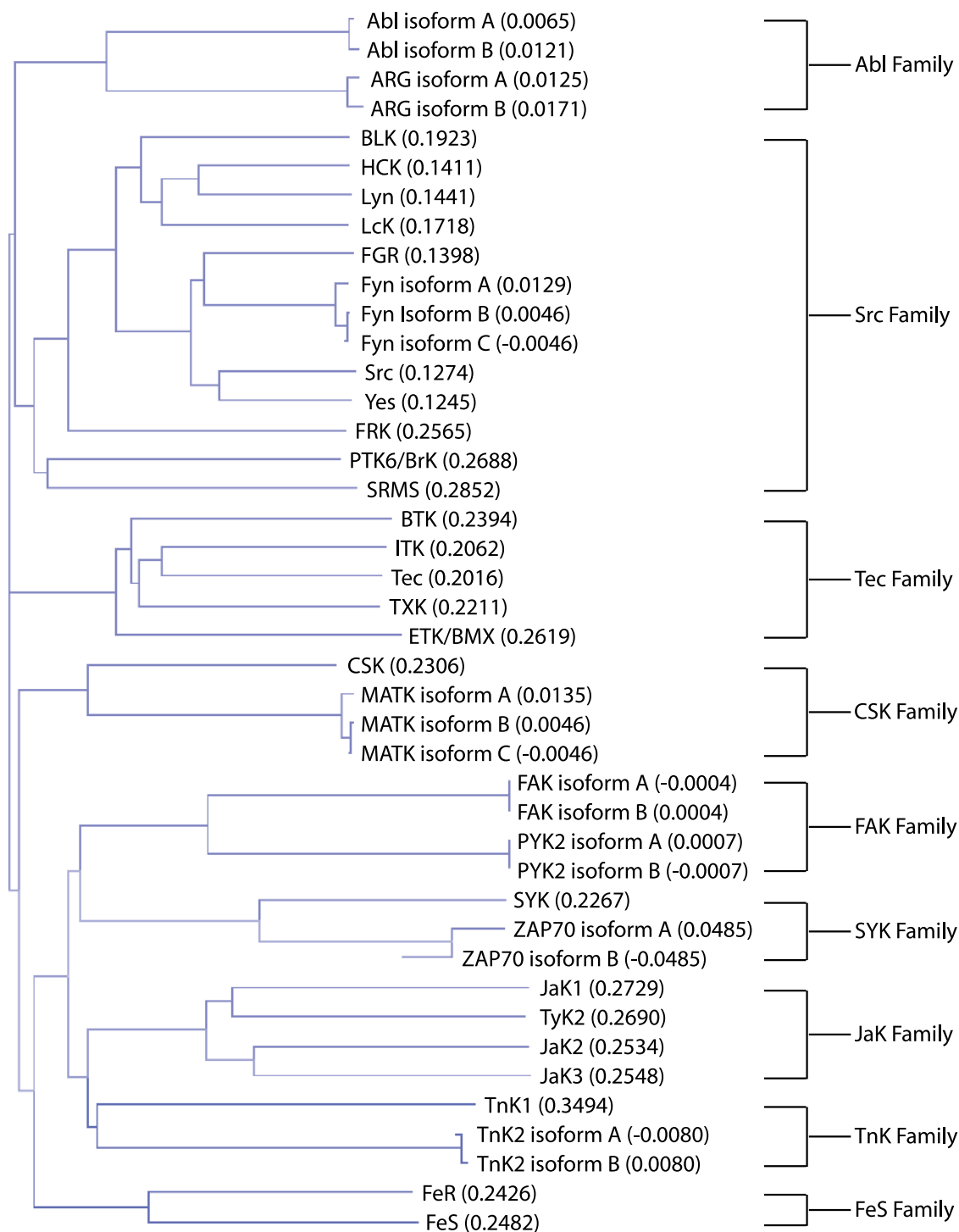
Received 29 October 2006; Revised 2 January 2007; Accepted 2 January 2007.

Copyright © 2007 Neoplasia Press, Inc. All rights reserved 1522-8002/07/\$25.00  
DOI 10.1593/neo.06694

of malignancies, including prostate cancer. In this review we will focus on each of the NRTKs and what is known about their respective roles in the biological processes of cell proliferation, migration, invasion, apoptosis, and angiogenesis in CaP.

There are several NRTK families. These are classified based on their structural similarities (Figure 1): Abl, tyrosine

kinase nonreceptor (TnK), C-terminal Src kinase (CSK), focal adhesion kinase (FAK), feline sarcoma oncogene/fujinami avian sarcoma viral oncogene homolog (FeS), Janus kinase (JaK), Src, Tec protein kinase (Tec), and spleen tyrosine kinase (SYK). Though these NRTK families are extensively and individually reviewed elsewhere, this



**Figure 1.** NRTK families and their members in a guide tree. Protein sequences are obtained from Entrez Gene and aligned using Vector NTI Advance software (Invitrogen, Carlsbad, CA). Vector NTI Advance uses the neighbor-joining method of phylogenetic tree construction by Saitou and Nei [127]. The numbers in parentheses after each kinase reflect the calculated distance values between pairs of analyzed sequences.

is the first time they are summarily discussed in relation to CaP.

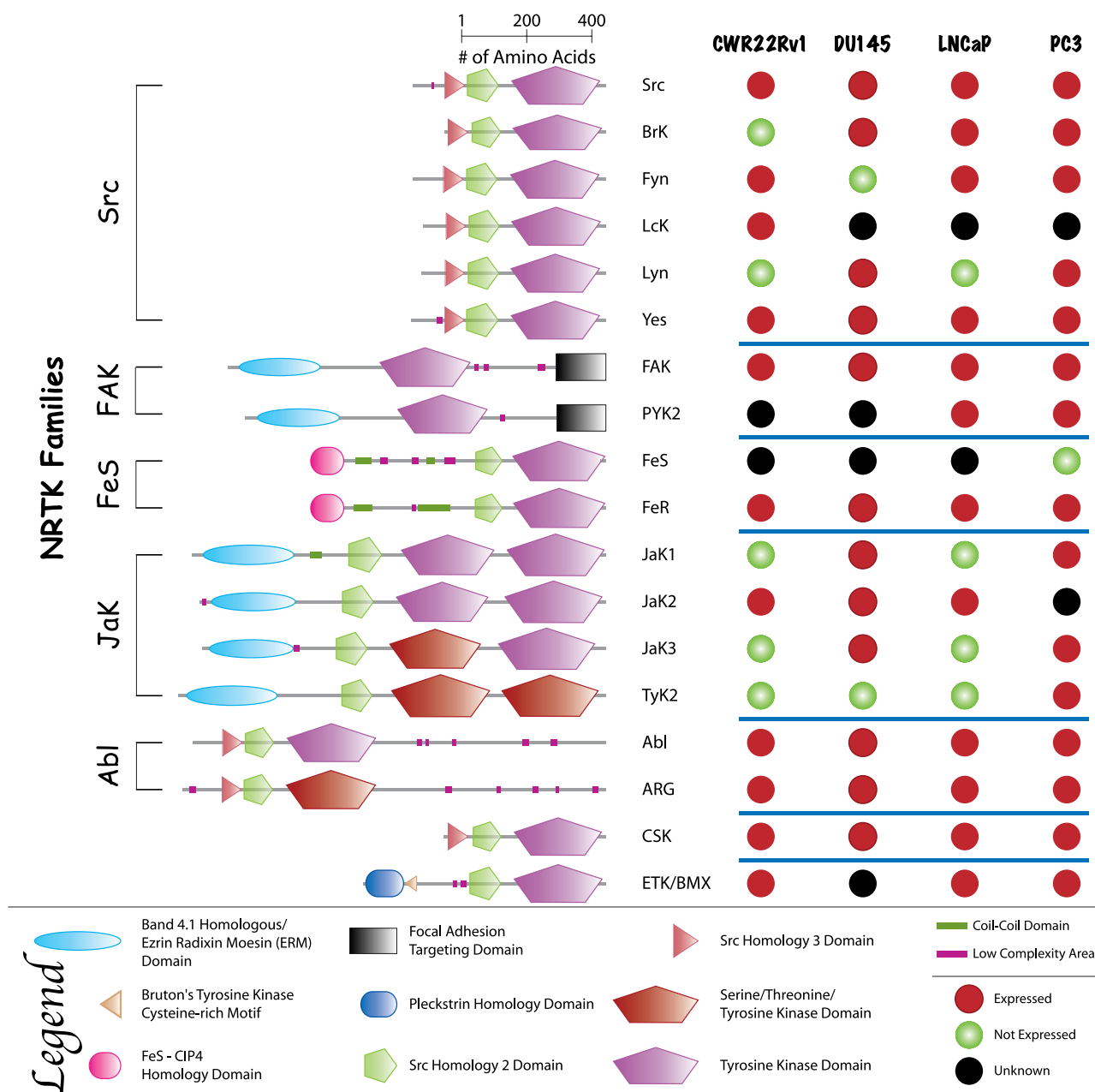
### Profiles of NRTKs in CaP

In 1996, Robinson et al. [3] led the first attempt at profiling the expression of TKs in CaP. Using a modified and improved reverse transcriptase–polymerase chain reaction approach, they identified nine NRTKs expressed in CWR22, a CaP xenograft. NRTKs include lymphocyte-specific protein tyrosine kinase (LcK), v-Yes-1 Yamaguchi sarcoma viral oncogene homolog 1(Yes), Abl, Abelson-related gene (*ARG*), Jak1, tyrosine kinase 2 (TyK2), and endothelial/epithelial tyrosine kinase/bone marrow X kinase (ETK/BMX). Fur-

thermore, *ARG* was found in several other CaP cell lines, which include PC-3, DU145, and LNCaP. In a similar study, Moore et al. [4] used degenerate polymerase chain reaction against conserved kinase catalytic subdomains and found that Abl, Jak1, Jak2, and TyK2 are expressed in surgically removed CaP tissues. In CWR22Rv1, DU145, LNCaP and PC3 cell lines, 18 NRTKs are expressed. This was confirmed by our internal data and also cross-referenced with several published reports (Figure 2).

### Src Family

As the first human proto-oncogene discovered, Src's history spans nearly a century and has been extensively reviewed [5–22]. Members of the Src family include B lymphoid



**Figure 2.** Summary of NRTK mRNA or protein expression in CWR22Rv1, DU145, LNCaP, and PC3 cell lines based on internal data and published reports. NRTK domain drawings and domain information were derived from Simple Modular Architecture Research Tool (SMART, Heidelberg, Germany).

tyrosine kinase (BLK), breast tumor kinase/protein tyrosine kinase 6 (BrK/PTK6), Gardner-Rasheed feline sarcoma viral oncogene homolog (FGR), Fyn oncogene related to Src, FGR, Yes (Fyn), hemopoietic cell kinase (HCK), LcK, v-Yes-1 Yamaguchi sarcoma viral-related oncogene homolog (Lyn), Src, Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites (SRMS), Yes, and Yes-related kinase (YRK). Of these, FGR, Fyn, LcK, Lyn, Src, and Yes are expressed in either CaP tumor samples or cell lines. Src, FGR, Fyn, LcK, and Lyn in particular have been the most widely studied in CaP.

**Src** The premier member of its namesake family, Src is extensively studied in cancer biology. Less is known, however, about Src biology in CaP. Though there are no published reports of Src expression or activation levels in clinical CaP specimens, Src is implicated in CaP through its association with factors that correlate positively with the presence or the progression of CaP disease, such as protein kinase C (PKC)  $\epsilon$ , endothelial-derived gene 1 (*EG-1*), and a truncated form of c-kit [23–25]. As further evidence of Src's possible involvement in CaP, DRS, a negative Src regulator, is down-regulated in CaP tissues and in prostate intraepithelial neoplasia relative to normal and benign prostate hyperplasia (BPH) tissues [26]. Thus, there is circumstantial clinical evidence that Src plays a role in CaP through its interactions with other factors of significance in CaP.

More is known about Src in CaP *in vitro*. Src is expressed in commonly used CaP cell lines CWR22Rv1, DU145, LAPC-4, LNCaP, and PC-3 (Figure 3). At first glance, Src protein expression levels in CaP cell lines do not positively correlate with the aggressiveness, AI state, or the proliferation rates of these cell lines. It is important to note, however, that wild-type cellular Src is not normally constitutively active. Its main role is to transduce signals of upstream activators. In cancer, the upstream signals may be aberrant, thus leading to improper activation of Src and its downstream pathways. One such pathway in CaP is Src activation by neuroendocrine ligands [27].

Neuroendocrine differentiation in CaP is theorized to be in part responsible for the development of AI CaP through the secretion of neuroendocrine ligands. There is evidence that

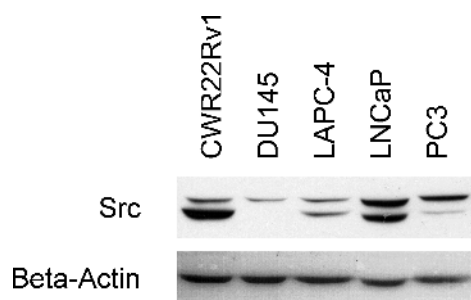
Src takes part in AI cell proliferation. Cyclic adenosine monophosphate (cAMP) analogs are able to activate Src following neuroendocrine differentiation, perhaps secondary to secreted neuroendocrine factors such as gastrin-releasing peptide and lysophosphatidic acid (LPA) [28–31]. LPA is thought to promote cell proliferation through the v-Ha-ras Harvey rat sarcoma viral oncogene homolog (Ras)–v-raf-1 murine leukemia viral oncogene homolog 1 (Raf)–ERK1/2 pathway in Src-dependent fashion. Bombesin, a *Xenopus* gastrin-releasing peptide homolog, can also activate ERK1/2 through Src, possibly through epidermal growth factor (EGF) receptor transactivation [32]. Once ERK1/2 has been activated, it can then activate the androgen receptor (AR) in an AI manner, which promotes cell growth [27,33]. In addition to LPA and bombesin, non-neurotrophic factors such as interleukin-8 (IL-8) and insulin-like growth factor-1 (IGF-1) also promote AI cell growth through Src [34,35].

In addition to cell proliferation, Src also takes part in antiapoptotic pathways in CaP. Bombesin, endothelin (ET1), met proto-oncogene (*Met*), and dihydrotestosterone-activated AR all inhibit apoptosis through Src activation [26,36–38]. There is, however, no consensus mechanism by which Src promotes cell survival. Nuclear factor  $\kappa$ B (NF- $\kappa$ B)–v-akt murine thymoma viral oncogene homolog 1 (Akt)–p21-associated kinase 1 (PAK1) pathway, MEK1/2–ERK1/2–CREB pathway, and signal and transducer of transcription 3 (STAT3)–dependent down-regulation of B-cell lymphoma leukemia (BCL-xL) and myeloid cell leukemia sequence 1 (MCL-1) are all pathways by which Src inhibits apoptosis [39].

Src is involved in other aspects of CaP biology: cell migration and adhesion. Src interacts with the extracellular signals through the IL-8 receptor, Met,  $\beta_1$  integrins, Kangai 1/cluster designation 82 (KAI1/CD82), and CD44 [23,34,40,41]. CD44 is a cell surface glycoprotein involved in cell–cell and cell–matrix adhesions. KAI1/CD82 functions as a metastasis suppressor, disrupting integrin-induced Src activation [42]. Intracellularly, Src modulates cell migration and adhesion through its interaction with FAK and p130 CRK-associated substrate (p130CAS) [2].

In addition to cell migration, Src also assists in tumor invasion through its regulation of matrix metalloproteinases (MMPs). MMPs aid in invasion through the degradation of the extracellular matrix. Bombesin promotes Src-dependent tumor progression and metastasis through the activation of MMP9 in conjunction with  $\beta_1$  integrins [43]. Src inhibition, on the other hand, decreases MMP9 activity levels [2,44].

Induction of angiogenesis by malignant cells is required for continued cell proliferation and metastasis, and vascular endothelial growth factor (VEGF) is a critical angiogenic factor. Src participates in angiogenesis in CaP through the Jak1–STAT3–VEGF pathway [45]. Src activation is also required for VEGF expression in simulated hypoxia environment through increased levels of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and activation of STAT3; as additional evidence of Src's involvement in angiogenesis, overexpression of active Src leads to increased VEGF expression [46]. Expression of the melanoma-differentiation–associated gene-7, a Src



**Figure 3.** Western blot analysis of total Src protein expression levels in prostate cancer cell lines. Src is shown as a doublet upon probing in most cell lines. Internal overexpression data (not shown) indicate that both bands are Src and that the doublet is not a result of nonspecific probing of other Src family kinase members.

inhibitor, on the other hand, inhibits the subsequent downstream STAT3–VEGF pathway [46,47].

Src is also of particular interest in CaP in part because of its interaction with steroid receptors. There is evidence that low amounts of AR and androgen lead to Src activation in the cytoplasm, thereby triggering downstream signaling events independent of AR's transcriptional and DNA-binding activity [38,48]. In fact, dominant negative Src can inhibit DNA synthesis following stimulation with low amounts of synthetic androgen. AR overexpression and higher concentrations of androgen, however, seem to bypass the Src pathway, leading to AR translocation to the nucleus and AR-transcriptional activity–based DNA synthesis.

In addition to the aforementioned activation of Src by androgen-activated AR, Src also associates with AR and estrogen receptor (ER) upon stimulation with estradiol, ultimately resulting in increased cell proliferation [38,49,50]. It is thought that Src serves as a scaffolding protein for the AR–ER complex. Steroidal ligand, however, is not necessary for AR–Src complex formation. Upon EGF stimulation, preformed heterodimers of ER $\alpha$  and AR form a complex with EGF receptor and Src, resulting in the activation and phosphorylation of EGF receptor, DNA synthesis, and cytoskeletal changes [51]. On the other hand, DOC-2/DAB2, a tumor suppressor and a negative Src regulator protein, is reported to inhibit AR's mitotic effects through the disruption of the AR–Src complex [52,53]. Thus, taken together with reports of AI AR activation by Src, AR and Src seem to be able to reciprocally transactivate, depending on the concentration and type of stimulatory ligand.

There are few published reports on cellular elements that negatively regulate Src in CaP. In addition to DOC-2/DAB2, tumor growth factor (TGF)  $\beta$  is reported to decrease both Src expression and its corresponding activity. This is shown by the accumulation of unphosphorylated form of SH2-containing protein (SHC) and a subsequent decrease in complex formation between SHC and growth factor receptor–bound protein 2 (Grb2) [54].

**BrK/PTK6** BrK is an Src family member, and little is known about it in CaP. In patient samples, BrK is detected in the nuclei of normal luminal epithelial tissues and well-differentiated tumors, but not in poorly differentiated tumors [55]. Localization of BrK in CaP cell lines LNCaP, which is poorly tumorigenic, and PC-3, which is more aggressive, is primarily nuclear and cytoplasmic, respectively. Though PC-3 expressed more BrK than LNCaP did, BrK is less active in PC-3 cells. Thus, the localization of BrK may play a role in the differentiation of CaP and its aggressiveness.

**FGR/Src-2** FGR is an Src kinase family member. It is a negative regulator of phosphatase and tensin homolog (PTEN) and a positive regulator of both Ras and Raf1, thus inhibiting apoptosis and stimulating cell growth, respectively [56]. Though little is known about FGR in CaP, FGR may be overexpressed in CaP, as shown by FGR DNA amplification in patient tumor tissues transitioning from androgen-

dependent to AI states [56]. Thus, FGR may play a role in CaP growth and survival.

**Fyn** Fyn is an Src family kinase member. It is involved in LNCaP mitogenesis following prolactin stimulation [57]. Though it is suggested that Fyn participates in prolactin-induced cell proliferation through K<sup>+</sup> ion channels, further studies are necessary in order to elucidate the mechanism of Fyn-modulated prolactin-induced cell proliferation in CaP.

**LcK** LcK is an Src family kinase member. It is expressed in CWR22 xenograft cells [3]. Little else is known about the role of LcK in CaP.

**Lyn** Lyn is an Src family kinase member expressed in normal prostate, 95% of primary CaP, and AI PC-3 and DU145 cells [58]. Lyn knockout mice have abnormal prostate gland development. Treatment with KRX-123, a Lyn-specific inhibitor, results in the inhibition of cell growth in DU145 and PC-3 cell lines. DU145 explants in mice treated with KRX-123 were found to also undergo apoptosis. Thus, Lyn seems to play a role in the proliferation and the apoptosis of CaP.

Lyn may also be an important regulator of cell migration in CaP. DU145 cells treated with dasatinib, an Src family kinase inhibitor, have reduced migratory activity [2]. On the other hand, Lyn can bind with neutral endopeptidase (NEP) and act as a competitive inhibitor to the PI3K–FAK complex, resulting in decreased cell migration [59]. Lyn's role in CaP cell migration is therefore inconclusive.

In CaP, Lyn is down-regulated by TGF $\beta$  and up-regulated by KAI1/CD82 [54,60]. Despite its elevated expression following KAI1/CD82 stimulation, however, Lyn's overall kinase activity was unchanged.

#### FAK Family

**FAK** As the predominate member of its namesake family of kinases, FAK is well studied in CaP. Several general reviews of FAK are available [61–71]. Though FAK may play roles in growth, apoptosis, and angiogenesis in CaP, FAK is known primarily for its role in cell motility and cytoskeletal rearrangement, as supported by *in vivo* and *in vitro* evidence. In clinical specimens, FAK expression and activation are uniformly higher in metastatic CaP than in normal and BPH tissues [72,73]. *In vitro* comparison between highly metastatic CaP cell lines and LNCaP, a cell line with lower metastatic potential, shows similar results, with increased expression and activation of FAK in the more aggressive cell lines [74]. FAK's association with molecular mediators of cell migration and adhesions are indicative of its function as well. Activated FAK complexes with  $\beta_1$  and  $\alpha(v)\beta_3$  integrins, molecules involved in cell adhesion [75–78]. As further evidence of FAK's function as a cell motility factor, inhibition of FAK with anti-FAK (pY397) antibody or FAK-related nonkinase (FRNK) resulted in significantly decreased cell migration [79].

Bombesin and IL-8 are both G protein–coupled receptors (GPCR) that activate FAK and stimulate cell migration

[34,79–81]. This is not surprising given FAK's reciprocal transactivation relationship with Src and both IL-8 and bombesin's abilities to activate Src. For bombesin to activate FAK, however, both PKC and an intact cytoskeleton are required [80,82]. Following its activation, FAK then phosphorylates p130CAS, leading to p130CAS–v-crk avian sarcoma virus CT10 oncogene homolog (CRKII) complex formation. Disruption of the p130CAS–CRKII complex by overexpressing KAI1/CD82 results in decreased cell motility [60].

Extracellularly, FAK is activated by integrins, ET1, bombesin, IL-8, and urokinase plasminogen activator (uPA), an invasion and metastasis factor in CaP [83,84]. Intracellularly, it is modulated by Src. It is important to note that Src and FAK activation often go hand in hand. They couple and reciprocally transactivate each other. There are, however, exceptions. FAK activation by autophosphorylation of tyrosine 397 is not Src-dependent; it is adhesion-dependent [74]. On the other hand, phosphorylation of tyrosine 861, which leads to increased FAK activity, is Src-dependent but not adhesion-dependent.

Though FAK is primarily a cell motility regulator, it is also involved in cell proliferation. Similar to cell migration, bombesin-induced FAK-mediated proliferation requires an intact cytoskeleton [80]. A signal downstream of FAK is ETK/BMX, an NRTK critical for bombesin-induced growth [27]. Following FAK activation of ETK/BMX, ETK/BMX subsequently activates AR, thereby inducing cell growth. Interestingly, not only can FAK indirectly activate AR, it can also be activated by membrane-associated AR in a PI3K-dependent manner [85].

In addition to migration and proliferation, FAK may also be involved in CaP angiogenesis and apoptosis. There is evidence that FAK induces VEGF transcription in an ERK1/2-dependent, Rap1-dependent, and Raf-dependent but Ras-independent manner [86]. Increased VEGF transcription may then lead to an increased level of its secreted protein and, thus, angiogenesis. In regard to apoptosis, treatment of cells with proapoptotic factors FTY720 and doxazosin both down-regulate FAK expression for reasons that are not currently known [87,88].

There are few known ways in which FAK is negatively regulated in CaP. Negative FAK regulators include *PTEN*, a tumor suppressor gene with dual phosphatase activity that is frequently deleted in aggressive CaP [89]. FAK may also be indirectly negatively regulated through the formation of the Lyn–PI3K–NEP complex instead of the PI3K–FAK complex [59].

**Proline-rich tyrosine kinase 2/cell adhesion kinase  $\beta$  (PYK2/CAK $\beta$ )** PYK2/CAK $\beta$  is a member of the FAK family of tyrosine kinases. A general review of PYK2 is available [90]. It is expressed in normal prostate epithelia and BPH, but its expression level decreases with increasing grade in CaP [91]. The gene is located on chromosome 8p21.1, a site of frequent deletion in CaP [92].

Though *in vivo* evidence suggests that PYK2 plays a tumor suppressive role in CaP, the *in vitro* evidence of this hypothesis is inconclusive. *In vitro* experiments show that

PYK2 is activated by LPA and tumor necrosis factor  $\alpha$ . PYK2 plays a role in the activation of ERK1/2 following LPA stimulation and may thus stimulate cell proliferation [93]. In addition, cells expressing dominant negative PYK2 have decreased proliferation rates. On the other hand, PYK2 indirectly inhibits AR activation through the inactivation of an AR-associated protein, ARA55 [94]. Thus, PYK2's role in CaP may depend on the androgen sensitivity status of the cells in question and requires further investigation and clarification.

#### FeS Family

The FeS family of NRTKs consists of two members: FeS/FpS and FpS/FeS–related tyrosine kinase (FeR). Little is known about the FeS family in CaP. An examination of CaP cell lines PC-3, PC133, and PC135 failed to detect FeS transcript [95]. FeR expression, on the other hand, was found in CaP cell lines PC-3, DU145, and LNCaP and positively correlated with CaP *versus* normal and BPH tissue samples [96]. Consistent with patient sample data, cells transfected with antisense FeR grew at a slower rate and were unable to grow in an anchorage-independent fashion. In the dog model, a higher FeR expression was found in dividing *versus* resting prostate epithelial cells and in cells displaying basal cell hyperplasia and metaplasia following postcastration estrogen treatment [96]. Thus, FeR is likely a proliferation factor in CaP.

#### JaK Family

**JaK1** The JaK family of kinases is well known for its role in signaling events in cells following cytokine stimulation and its association with the STAT family of kinases. Though JaK1 is present in some clinical CaP specimens, JaK1 is reported to be either negatively regulated or mutated in many CaP cell lines [4,97,98]. LNCaP is found to have both nonsense mutation and repressed JaK1 transcription whereas CWR22Rv1 and LAPC-4 have only nonsense mutations and no known transcriptional repression.

In DU145 cells, which have wild-type JaK1, there are reports that JaK1 associates with breast cancer susceptibility gene 1 (*BRCA1*) [99]. When *BRCA1* is overexpressed, JaK1 and STAT3 become activated. Subsequent inhibition of STAT3 activation results in decreased cell proliferation as well as in apoptosis. Interestingly, inhibition of JaK1 in wild-type DU145 does not result in apoptosis [100]. Thus, it may be possible that although JaK1 activation by *BRCA1* leads to increased JaK1 and STAT3 activation, STAT3 may in fact not be directly downstream of JaK1 in CaP, and their concurrent activation is coincidental.

JaK1 may also play a role in the inhibition of CaP migration and invasion following IL-10 stimulation [101]. Tissue inhibitor of metalloproteinases (TIMP) 1 is an anti-invasion factor. IL-10 is known to activate the JaK1–IL-10E1–TIMP-1 pathway in CaP [102].

**JaK2** JaK2 is expressed in some CaP tissues [4]. Similar to JaK1, JaK2 is also activated by *BRCA1* in DU145 cells [99]. It

is interesting to note that although Jak1 inhibition does not result in apoptosis in wild-type DU145 cells, inhibition of Jak2 does [100]. Thus, STAT3 activation in DU145 may be dependent on Jak2 rather than on Jak1. Whether STAT3 is activated by Jak1 or Jak2 in CaP, however, seems to be cell line-dependent [103].

**Jak2** may also be involved in cell proliferation in CaP. Tyrosine kinase inhibitor peptide (TKIP) directly inhibits Jak2 autophosphorylation, decreases STAT3 activation, and slows CaP proliferation [104]. Consistent with decreased cell proliferation and STAT3 activation, cyclin D1 level is decreased and cells are arrested in the G<sub>1</sub> phase of the cell cycle following TKIP treatment. Thus, Jak2 may be important for CaP growth through the STAT3 pathway. In addition to STAT3, Jak2 may be of particular importance in CaP through its regulation of STAT5, a factor that positively correlates with the histological grade of CaP [105,106].

**Tyk2** Tyk2 is expressed in some CaP tissues [4]. Though Tyk2 may also be involved in CaP migration and invasion and similarly participates in the activation of IL-10E1 following IL-10 stimulation of CaP cells as Jak1, its temporal regulation profile is different from that of Jak1 [101,102].

#### Members of Other NRTK Families

**Abl** Abl is well known for its role in the etiology of CML following the formation of the Philadelphia chromosome (t(9;22)) and the breakpoint cluster region (Bcr)–Abl hybrid gene product. Less is known, however, about Abl in CaP. It is known that Abl is expressed in some CaP specimens and that Abl is necessary for retinoblastoma-mediated  $\gamma$ -radiation-induced apoptosis in DU145 cells [4,107]. There is indirect evidence that Abl may be important in CaP. Human spectrin SH domain-binding protein 1 (*Hssh3bp1*) is a gene that binds to Abl, possibly as a negative regulator [108]. A majority (9 of 17) of CaP tumor samples analyzed failed to express *Hssh3bp1*. Furthermore, *Hssh3bp1* is found on chromosome 10p, a region frequently deleted in CaP. Thus, Abl may be circumstantially implicated in CaP through its association with *Hssh3bp1*.

Imatinib mesylate (Gleevec; Novartis, East Hanover, NJ) is a Bcr–Abl inhibitor that is clinically used for the treatment of CML. It also has activity against Kit kinase and platelet-derived growth factor (PDGF) receptor. *In vitro*, Gleevec inhibits CaP cell growth with IC<sub>50</sub> in the 10- $\mu$ M range [109]. In mice models, however, Gleevec's efficacy against CaP growth is inconclusive with some, but not all, studies showing growth inhibition [110–113].

Similarly, preliminary results from clinical studies also paint a mixed picture. A phase I clinical trial of Gleevec in combination with docetaxel in AI CaP showed a prostate-specific antigen (PSA) decline in 14 of 21 patients, although it is unknown whether the decline can be attributed to Gleevec or docetaxel [114]. In another AI CaP study, Gleevec in combination with zoledronic acid (Zometa, Novartis) showed no clinical effect in 15 CaP patients [115]. Lastly, as monotherapy in 16 patients with androgen-sensitive CaP, Gleevec treatment resulted in nine patients with stable PSA levels

and seven patients with PSA progression [116]. Thus, clinical use of Gleevec as monotherapy in CaP may be ineffective. The efficacy of using Gleevec as an adjuvant therapy to other treatment modalities is presently unknown.

**CSK** CSK is a well known negative Src regulator [117]. Little is directly known about CSK in CaP other than that it complexes with FAK in metastatic tumors and PC-3 cells [73].

**ETK/BMX** Discovered in 1994, ETK/BMX belongs to the Tec family of NRTK [118]. In CaP, ETK is downstream of PI3K in the induction of the neuroendocrine differentiation of LNCaP cells following IL-6 stimulation [119]. It is also reported to function as an antiapoptotic factor. Overexpression of ETK confers resistance to apoptosis in CaP cells through its interaction with PI3K [120]. PI3K is not, however, required for ETK activation [27]. Another mechanism by which ETK may protect against apoptosis is through its interaction with p53 [121]. Interestingly, ETK also participates in the apoptotic cascade in CaP cells. Introduction of ETK's C-terminal fragment into PC-3 cells can lead to apoptosis following proteolytic cleavage of ETK by caspases [122].

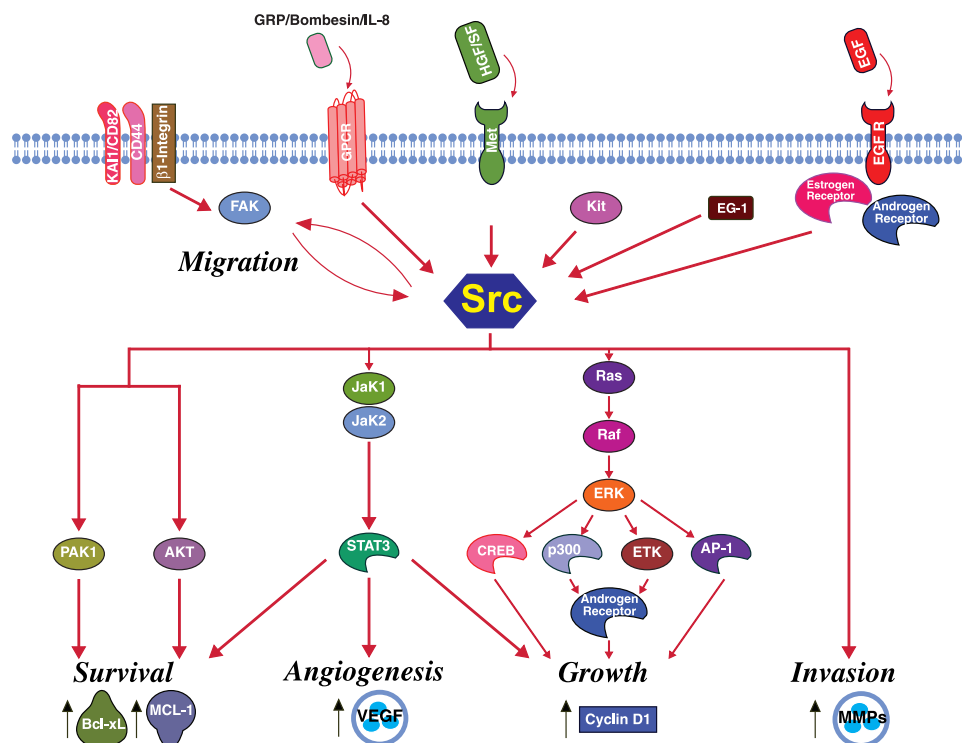
ETK is also critical for cell proliferation following bombesin stimulation and AR activation in CaP [27]. ETK serves as a signal transducer between Src and FAK upstream and AR downstream. ETK alone, however, is insufficient for AR activation. ETK must be able to reciprocally transactivate with Pim1 before AR activation [123,124].

**Other NRTKS** SYK and TNK1 are other NRTKs that have been studied in CaP. Virtually nothing is known about their properties and functions in prostate cancer except that the promoter region of SYK is hypermethylated and TNK1 transcript is found in prostate tissues [125,126]. SYK expression may thus be down-regulated in CaP, whereas TNK1 protein expression level remains to be investigated.

#### Conclusion

Much is known regarding specific NRTKs in CaP (Src, FAK, Jak1/2, and ETK), whereas less is known about the other NRTKs. Perhaps it is not a coincidence that the well-studied Src, FAK, Jak1/2, and ETK kinases are involved in processes indispensable to the pathology of CaP: cell growth, migration, invasion, angiogenesis, and apoptosis. It is therefore imperative that we learn more about these NRTKs through future studies. Although Src, FAK, Jak1/2, and ETK are important in CaP biology, we should not neglect the other NRTKs that may also play important roles in CaP and should also investigate the lesser known NRTKs.

Looking at the current literature of NRTKs in CaP, there emerges a picture of Src being an ubiquitous player in multiple biological processes interacting with numerous players in multiple signaling pathways. Src transduces signals from upstream receptors such as IL-8, EGF, IGF-1, neurotensin, ET1, and HGF/SF to downstream molecules such as FAK, ETK, JAK1/2, STAT3, Ras, ERK1/2, Akt, HIF-1 $\alpha$ , and, of particular significance in CaP biology, AR (Figure 4). Given



**Figure 4.** Known Src pathways in prostate cancer. The close proximity of molecules not connected with arrows denote physical association. Red arrows denote activation. Black arrows denote change in levels of molecule. Figure templates were provided by BioCarta (San Diego, CA).

the preponderance of evidence in multiple biological processes linking Src to CaP, Src is likely an important point of pathway convergence in CaP. Perhaps it is not surprising then that Src is currently the only NRTK target in clinical trials for CaP, whereas no NRTK-specific therapy is available for general clinical use in CaP. What remains unclear, however, is Src's relative importance in particular stages of CaP: oncogenesis, growth, survival, AI growth, angiogenesis, and metastasis. Nevertheless, with cancer treatments moving toward targeting specific pathways, it is important that we continue investigating signaling pathways so that we can develop novel therapies through continued research.

## References

- [1] Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, and Thun MJ (2006). Cancer statistics, 2006. *CA Cancer J Clin* **56**, 106–130.
- [2] Nam S, Kim D, Cheng JQ, Zhang S, Lee JH, Buettner R, Mirosevich J, Lee FY, and Jove R (2005). Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res* **65**, 9185–9189.
- [3] Robinson D, He F, Pretlow T, and Kung HJ (1996). A tyrosine kinase profile of prostate carcinoma. *Proc Natl Acad Sci USA* **93**, 5958–5962.
- [4] Moore TM, Garg R, Johnson C, Coptcoat MJ, Ridley AJ, and Morris JD (2000). PSK, a novel STE20-like kinase derived from prostatic carcinoma that activates the c-Jun N-terminal kinase mitogen-activated protein kinase pathway and regulates actin cytoskeletal organization. *J Biol Chem* **275**, 4311–4322.
- [5] Boggan TJ and Eck MJ (2004). Structure and regulation of Src family kinases. *Oncogene* **23**, 7918–7927.
- [6] Bromann PA, Korkaya H, and Courtneidge SA (2004). The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23**, 7957–7968.
- [7] Gauld SB and Cambier JC (2004). Src-family kinases in B-cell development and signaling. *Oncogene* **23**, 8001–8006.
- [8] Geahlen RL, Handley MD, and Harrison ML (2004). Molecular inter-diction of Src-family kinase signaling in hematopoietic cells. *Oncogene* **23**, 8024–8032.
- [9] Kalia LV, Gingrich JR, and Salter MW (2004). Src in synaptic transmission and plasticity. *Oncogene* **23**, 8007–8016.
- [10] Luttrell DK and Luttrell LM (2004). Not so strange bedfellows: G-protein–coupled receptors and Src family kinases. *Oncogene* **23**, 7969–7978.
- [11] Martin GS (2004). The road to Src. *Oncogene* **23**, 7910–7917.
- [12] Palacios EH and Weiss A (2004). Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* **23**, 7990–8000.
- [13] Parsons SJ and Parsons JT (2004). Src family kinases, key regulators of signal transduction. *Oncogene* **23**, 7906–7909.
- [14] Playford MP and Schaller MD (2004). The interplay between Src and integrins in normal and tumor biology. *Oncogene* **23**, 7928–7946.
- [15] Reynolds AB and Rocznik-Ferguson A (2004). Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene* **23**, 7947–7956.
- [16] Shupnik MA (2004). Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* **23**, 7979–7989.
- [17] Silva CM (2004). Role of STATs as downstream signal transducers in Src family kinase–mediated tumorigenesis. *Oncogene* **23**, 8017–8023.
- [18] Alvarez RH, Kantarjian HM, and Cortes JE (2006). The role of Src in solid and hematologic malignancies: development of new-generation Src inhibitors. *Cancer* **107**, 1918–1929.
- [19] Trevino JG, Summy JM, and Gallick GE (2006). SRC inhibitors as potential therapeutic agents for human cancers. *Mini Rev Med Chem* **6**, 681–687.
- [20] Summy JM and Gallick GE (2006). Treatment for advanced tumors: SRC reclaims center stage. *Clin Cancer Res* **12**, 1398–1401.
- [21] Alper O and Bowden ET (2005). Novel insights into c-Src. *Curr Pharm Des* **11**, 1119–1130.
- [22] Roskoski R Jr (2005). Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* **331**, 1–14.
- [23] Wu D, Thakore CU, Wescott GG, McCubrey JA, and Terrian DM (2004). Integrin signaling links protein kinase C epsilon to the protein kinase B/Akt survival pathway in recurrent prostate cancer cells. *Oncogene* **23**, 8659–8672.
- [24] Paronetto MP, Farini D, Sammarco I, Maturo G, Vespasiani G, Geremia R, Rossi P, and Sette C (2004). Expression of a truncated

- form of the c-Kit tyrosine kinase receptor and activation of Src kinase in human prostatic cancer. *Am J Pathol* **164**, 1243–1251.
- [25] Lu M, Zhang L, Maul RS, Sartippour MR, Norris A, Whitelegge J, Rao JY, and Brooks MN (2005). The novel gene *EG-1* stimulates cellular proliferation. *Cancer Res* **65**, 6159–6166.
  - [26] Kim CJ, Shimakage M, Kushima R, Mukaisho K, Shinka T, Okada Y, and Inoue H (2003). Down-regulation of drs mRNA in human prostate carcinomas. *Hum Pathol* **34**, 654–657.
  - [27] Lee LF, Guan J, Qiu Y, and Kung HJ (2001). Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. *Mol Cell Biol* **21**, 8385–8397.
  - [28] Allard P, Beaulieu P, Aprikian A, and Chevalier S (2000). Bombesin modulates the association of Src with a nuclear 110-kD protein expressed in dividing prostate cells. *J Androl* **21**, 367–375.
  - [29] Daaka Y (2002). Mitogenic action of LPA in prostate. *Biochim Biophys Acta* **1582**, 265–269.
  - [30] Kue PF and Daaka Y (2000). Essential role for G proteins in prostate cancer cell growth and signaling. *J Urol* **164**, 2162–2167.
  - [31] Bang YJ, Pirnia F, Fang WG, Kang WK, Sartor O, Whitesell L, Ha MJ, Tsokos M, Sheahan MD, Nguyen P, et al. (1994). Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. *Proc Natl Acad Sci USA* **91**, 5330–5334.
  - [32] Xiao D, Qu X, and Weber HC (2003). Activation of extracellular signal-regulated kinase mediates bombesin-induced mitogenic responses in prostate cancer cells. *Cell Signal* **15**, 945–953.
  - [33] Gong J, Zhu J, Goodman OB, Pestell RG, Schlegel PN, Nanus DM, and Shen R (2006). Activation of p300 histone acetyltransferase activity and acetylation of the androgen receptor by bombesin in prostate cancer cells. *Oncogene*.
  - [34] Lee LF, Louie MC, Desai SJ, Yang J, Chen HW, Evans CP, and Kung HJ (2004). Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene* **23**, 2197–2205.
  - [35] Pandini G, Mineo R, Frasca F, Roberts CT Jr, Marcelli M, Vigneri R, and Belfiore A (2005). Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. *Cancer Res* **65**, 1849–1857.
  - [36] Fan S, Gao M, Meng Q, Laterra JJ, Symons MH, Coniglio S, Pestell RG, Goldberg ID, and Rosen EM (2005). Role of NF-kappaB signaling in hepatocyte growth factor/scatter factor-mediated cell protection. *Oncogene*.
  - [37] Sumitomo M, Shen R, Goldberg JS, Dai J, Navarro D, and Nanus DM (2000). Neutral endopeptidase promotes phorbol ester-induced apoptosis in prostate cancer cells by inhibiting neuropeptide-induced protein kinase C delta degradation. *Cancer Res* **60**, 6590–6596.
  - [38] Unni E, Sun S, Nan B, McPhaul MJ, Cheskis B, Mancini MA, and Marcelli M (2004). Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res* **64**, 7156–7168.
  - [39] Kotha A, Sekharam M, Cilenti L, Siddiquee K, Khaled A, Zervos AS, Carter B, Turkson J, and Jove R (2006). Resveratrol inhibits Src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein. *Mol Cancer Ther* **5**, 621–629.
  - [40] Zhu D and Bourguignon LY (1998). The ankyrin-binding domain of CD44s is involved in regulating hyaluronic acid-mediated functions and prostate tumor cell transformation. *Cell Motil Cytoskeleton* **39**, 209–222.
  - [41] Jee B, Jin K, Hahn JH, Song HG, and Lee H (2003). Metastasis-suppressor KAI1/CD82 induces homotypic aggregation of human prostate cancer cells through Src-dependent pathway. *Exp Mol Med* **35**, 30–37.
  - [42] Sridhar SC and Miranti CK (2006). Tetraspanin KAI1/CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases. *Oncogene* **25**, 2367–2378.
  - [43] Festuccia C, Angelucci A, Gravina G, Eleuterio E, Vicentini C, and Bologna M (2002). Bombesin-dependent pro-MMP-9 activation in prostatic cancer cells requires beta<sub>1</sub> integrin engagement. *Exp Cell Res* **280**, 1–11.
  - [44] Recchia I, Rucci N, Festuccia C, Bologna M, MacKay AR, Migliaccio S, Longo M, Susa M, Fabbro D, and Teti A (2003). Pyrrolopyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells *in vitro*. *Eur J Cancer* **39**, 1927–1935.
  - [45] Nam S, Buettner R, Turkson J, Kim D, Cheng JQ, Muehlbauer S, Hippe F, Vatter S, Merz KH, Eisenbrand G, et al. (2005). Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc Natl Acad Sci USA* **102**, 5998–6003.
  - [46] Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, and Gallick GE (2005). HIF-1alpha, STAT3, CBP/p300 and Ref-1/ARE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. *Oncogene* **24**, 3110–3120.
  - [47] Inoue S, Branch CD, Gallick GE, Chada S, and Ramesh R (2005). Inhibition of Src kinase activity by Ad-mda7 suppresses vascular endothelial growth factor expression in prostate carcinoma cells. *Mol Ther* **12**, 707–715.
  - [48] Castoria G, Lombardi M, Barone MV, Bilancio A, Di Domenico M, De Falco A, Varricchio L, Bottero D, Nanayakkara M, Migliaccio A, et al. (2004). Rapid signalling pathway activation by androgens in epithelial and stromal cells. *Steroids* **69**, 517–522.
  - [49] Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, et al. (2000). Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *EMBO J* **19**, 5406–5417.
  - [50] Chieffi P, Kisslinger A, Sinisi AA, Abbondanza C, and Tramontano D (2003). 17beta-Estradiol-induced activation of ERK1/2 through endogenous androgen receptor-estradiol receptor alpha-Src complex in human prostate cells. *Int J Oncol* **23**, 797–801.
  - [51] Migliaccio A, Di Domenico M, Castoria G, Nanayakkara M, Lombardi M, de Falco A, Bilancio A, Varricchio L, Ciociola A, and Auricchio F (2005). Steroid receptor regulation of epidermal growth factor signaling through Src in breast and prostate cancer cells: steroid antagonist action. *Cancer Res* **65**, 10585–10593.
  - [52] Zhou J, Hernandez G, Tu SW, Huang CL, Tseng CP, and Hsieh JT (2005). The role of DOC-2/DAB2 in modulating androgen receptor-mediated cell growth via the nongenomic c-Src-mediated pathway in normal prostatic epithelium and cancer. *Cancer Res* **65**, 9906–9913.
  - [53] Zhou J, Scholes J, and Hsieh JT (2003). Characterization of a novel negative regulator (DOC-2/DAB2) of c-Src in normal prostatic epithelium and cancer. *J Biol Chem* **278**, 6936–6941.
  - [54] Atfi A, Drobetsky E, Boissonneault M, Chapdelaine A, and Chevalier S (1994). Transforming growth factor beta down-regulates Src family protein tyrosine kinase signaling pathways. *J Biol Chem* **269**, 30688–30693.
  - [55] Derry JJ, Prins GS, Ray V, and Tyner AL (2003). Altered localization and activity of the intracellular tyrosine kinase Brk/Sik in prostate tumor cells. *Oncogene* **22**, 4212–4220.
  - [56] Edwards J, Krishna NS, Witton CJ, and Bartlett JM (2003). Gene amplifications associated with the development of hormone-resistant prostate cancer. *Clin Cancer Res* **9**, 5271–5281.
  - [57] Van Coppenolle F, Skryma R, Ouadid-Ahidouch H, Slomianky C, Roudbaraki M, Delcourt P, Dewailly E, Humez S, Crepin A, Gourdou I, et al. (2004). Prolactin stimulates cell proliferation through a long form of prolactin receptor and K<sup>+</sup> channel activation. *Biochem J* **377**, 569–578.
  - [58] Goldenberg-Furmanov M, Stein I, Pikarsky E, Rubin H, Kasem S, Wygoda M, Weinstein I, Reuveni H, and Ben-Sasson SA (2004). Lyn is a target gene for prostate cancer: sequence-based inhibition induces regression of human tumor xenografts. *Cancer Res* **64**, 1058–1066.
  - [59] Sumitomo M, Shen R, Walburg M, Dai J, Geng Y, Navarro D, Boileau G, Papandreou CN, Giancotti FG, Knudsen B, et al. (2000). Neutral endopeptidase inhibits prostate cancer cell migration by blocking focal adhesion kinase signaling. *J Clin Invest* **106**, 1399–1407.
  - [60] Zhang XA, He B, Zhou B, and Liu L (2003). Requirement of the p130CAS-Crk coupling for metastasis suppressor KAI1/CD82-mediated inhibition of cell migration. *J Biol Chem* **278**, 27319–27328.
  - [61] Wozniak MA, Modzelewska K, Kwong L, and Keely PJ (2004). Focal adhesion regulation of cell behavior. *Biochim Biophys Acta* **1692**, 103–119.
  - [62] Cohen LA and Guan JL (2005). Mechanisms of focal adhesion kinase regulation. *Curr Cancer Drug Targets* **5**, 629–643.
  - [63] McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, and Frame MC (2005). The role of focal-adhesion kinase in cancer — a new therapeutic opportunity. *Nat Rev Cancer* **5**, 505–515.
  - [64] Mitra SK, Hanson DA, and Schlaepfer DD (2005). Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* **6**, 56–68.
  - [65] Gabarra-Niecko V, Schaller MD, and Dunty JM (2003). FAK regulates biological processes important for the pathogenesis of cancer. *Cancer Metastasis Rev* **22**, 359–374.
  - [66] Hanks SK, Ryzhova L, Shin NY, and Brabek J (2003). Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci* **8**, d982–d996.

- [67] Hauck CR, Hsia DA, and Schlaepfer DD (2002). The focal adhesion kinase—a regulator of cell migration and invasion. *IUBMB Life* **53**, 115–119.
- [68] Hecker TP and Gladson CL (2003). Focal adhesion kinase in cancer. *Front Biosci* **8**, s705–s714.
- [69] McLean GW, Avizienyte E, and Frame MC (2003). Focal adhesion kinase as a potential target in oncology. *Expert Opin Pharmacother* **4**, 227–234.
- [70] Parsons JT (2003). Focal adhesion kinase: the first ten years. *J Cell Sci* **116**, 1409–1416.
- [71] Schaller MD (2001). Biochemical signals and biological responses elicited by the focal adhesion kinase. *Biochim Biophys Acta* **1540**, 1–21.
- [72] Rovin JD, Frierson HF Jr, Ledin W, Parsons JT, and Adams RB (2002). Expression of focal adhesion kinase in normal and pathologic human prostate tissues. *Prostate* **53**, 124–132.
- [73] Tremblay L, Hauck W, Aprikian AG, Begin LR, Chapdelaine A, and Chevalier S (1996). Focal adhesion kinase (pp125FAK) expression, activation and association with paxillin and p50CSK in human metastatic prostate carcinoma. *Int J Cancer* **68**, 164–171.
- [74] Slack JK, Adams RB, Rovin JD, Bissonette EA, Stoker CE, and Parsons JT (2001). Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. *Oncogene* **20**, 1152–1163.
- [75] Bergan R, Kyle E, Nguyen P, Trepel J, Ingui C, and Neckers L (1996). Genistein-stimulated adherence of prostate cancer cells is associated with the binding of focal adhesion kinase to beta-1-integrin. *Clin Exp Metastasis* **14**, 389–398.
- [76] Zheng DQ, Woodard AS, Fornaro M, Tallini G, and Languino LR (1999). Prostatic carcinoma cell migration via alpha(v)beta<sub>3</sub> integrin is modulated by a focal adhesion kinase pathway. *Cancer Res* **59**, 1655–1664.
- [77] Zheng DQ, Woodard AS, Tallini G, and Languino LR (2000). Substrate specificity of alpha(v)beta(3) integrin-mediated cell migration and phosphatidylinositol 3-kinase/AKT pathway activation. *J Biol Chem* **275**, 24565–24574.
- [78] Bello-DeOcampo D, Kleinman HK, DeOcampo ND, and Webber MM (2001). Laminin-1 and alpha<sub>6</sub>beta<sub>1</sub> integrin regulate acinar morphogenesis of normal and malignant human prostate epithelial cells. *Prostate* **46**, 142–153.
- [79] Lacoste J, Aprikian AG, and Chevalier S (2005). Focal adhesion kinase is required for bombesin-induced prostate cancer cell motility. *Mol Cell Endocrinol* **235**, 51–61.
- [80] Duncan MD, Harmon JW, and Duncan LK (1996). Actin disruption inhibits bombesin stimulation of focal adhesion kinase (pp125FAK) in prostate carcinoma. *J Surg Res* **63**, 359–363.
- [81] Aprikian AG, Tremblay L, Han K, and Chevalier S (1997). Bombesin stimulates the motility of human prostate-carcinoma cells through tyrosine phosphorylation of focal adhesion kinase and of integrin-associated proteins. *Int J Cancer* **72**, 498–504.
- [82] Liu Y, Kyle E, Lieberman R, Crowell J, Kelloff G, and Bergan RC (2000). Focal adhesion kinase (FAK) phosphorylation is not required for genistein-induced FAK-beta-1-integrin complex formation. *Clin Exp Metastasis* **18**, 203–212.
- [83] Margheri F, D'Alessio S, Serrati S, Pucci M, Annunziato F, Cosmi L, Liotta F, Angeli R, Angelucci A, Gravina GL, et al. (2005). Effects of blocking urokinase receptor signaling by antisense oligonucleotides in a mouse model of experimental prostate cancer bone metastases. *Gene Ther*.
- [84] Sabbisetti V, Chigurupati S, Thomas S, and Shah G (2006). Calcitonin stimulates the secretion of urokinase-type plasminogen activator from prostate cancer cells: its possible implications on tumor cell invasion. *Int J Cancer* **118**, 2694–2702.
- [85] Papakonstanti EA, Kampa M, Castanas E, and Stournaras C (2003). A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. *Mol Endocrinol* **17**, 870–881.
- [86] Sheta EA, Harding MA, Conaway MR, and Theodorescu D (2000). Focal adhesion kinase, Rap1, and transcriptional induction of vascular endothelial growth factor. *J Natl Cancer Inst* **92**, 1065–1073.
- [87] Permpongkosol S, Wang JD, Takahara S, Matsumiya K, Nonomura N, Nishimura K, Tsujimura A, Kongkanand A, and Okuyama A (2002). Anticarcinogenic effect of FTY720 in human prostate carcinoma DU145 cells: modulation of mitogenic signaling, FAK, cell-cycle entry and apoptosis. *Int J Cancer* **98**, 167–172.
- [88] Walden PD, Globina Y, and Nieder A (2004). Induction of anoikis by doxazosin in prostate cancer cells is associated with activation of caspase-3 and a reduction of focal adhesion kinase. *Urol Res* **32**, 261–265.
- [89] Ittmann MM (1998). Chromosome 10 alterations in prostate adenocarcinoma (review). *Oncol Rep* **5**, 1329–1335.
- [90] Gelman IH (2003). Pyk 2 FAKs, any two FAKs. *Cell Biol Int* **27**, 507–510.
- [91] Stanzione R, Picascia A, Chieffi P, Imbimbo C, Palmieri A, Mirone V, Staibano S, Franco R, De Rosa G, Schlessinger J, et al. (2001). Variations of proline-rich kinase Pyk2 expression correlate with prostate cancer progression. *Lab Invest* **81**, 51–59.
- [92] Inazawa J, Sasaki H, Nagura K, Kakazu N, Abe T, and Sasaki T (1996). Precise localization of the human gene encoding cell adhesion kinase beta (CAK beta/PYK2) to chromosome 8 at p21.1 by fluorescence *in situ* hybridization. *Hum Genet* **98**, 508–510.
- [93] Picascia A, Stanzione R, Chieffi P, Kisslinger A, Dikic I, and Tramontano D (2002). Proline-rich tyrosine kinase 2 regulates proliferation and differentiation of prostate cells. *Mol Cell Endocrinol* **186**, 81–87.
- [94] Wang X, Yang Y, Guo X, Sampson ER, Hsu CL, Tsai MY, Yeh S, Wu G, Guo Y, and Chang C (2002). Suppression of androgen receptor transactivation by Pyk2 via interaction and phosphorylation of the ARA55 coregulator. *J Biol Chem* **277**, 15426–15431.
- [95] Rijnders AW, van der Korput JA, van Steenbrugge GJ, Romijn JC, and Trapman J (1985). Expression of cellular oncogenes in human prostatic carcinoma cell lines. *Biochem Biophys Res Commun* **132**, 548–554.
- [96] Allard P, Zoubeydi A, Nguyen LT, Tessier S, Tanguay S, Chevrete M, Aprikian A, and Chevalier S (2000). Links between Fer tyrosine kinase expression levels and prostate cell proliferation. *Mol Cell Endocrinol* **159**, 63–77.
- [97] Dunn GP, Sheehan KC, Old LJ, and Schreiber RD (2005). IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression. *Cancer Res* **65**, 3447–3453.
- [98] Rossi MR, Hawthorn L, Platt J, Burkhardt T, Cowell JK, and Ionov Y (2005). Identification of inactivating mutations in the JAK1, SYNJ2, and CLPTM1 genes in prostate cancer cells using inhibition of nonsense-mediated decay and microarray analysis. *Cancer Genet Cytogenet* **161**, 97–103.
- [99] Gao B, Shen X, Kunos G, Meng Q, Goldberg ID, Rosen EM, and Fan S (2001). Constitutive activation of JAK-STAT3 signaling by BRCA1 in human prostate cancer cells. *FEBS Lett* **488**, 179–184.
- [100] Barton BE, Karras JG, Murphy TF, Barton A, and Huang HF (2004). Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: direct STAT3 inhibition induces apoptosis in prostate cancer lines. *Mol Cancer Ther* **3**, 11–20.
- [101] Stearns ME, Wang M, Hu Y, and Garcia FU (2003). Interleukin-10 activation of the interleukin-10E1 pathway and tissue inhibitor of metalloproteinase-1 expression is enhanced by proteasome inhibitors in primary prostate tumor lines. *Mol Cancer Res* **1**, 631–642.
- [102] Wang M, Hu Y, and Stearns ME (2003). A novel IL-10 signalling mechanism regulates TIMP-1 expression in human prostate tumour cells. *Br J Cancer* **88**, 1605–1614.
- [103] Barton BE, Murphy TF, Adem P, Watson RA, Irwin RJ, and Huang HF (2001). IL-6 signaling by STAT3 participates in the change from hyperplasia to neoplasia in NRP-152 and NRP-154 rat prostatic epithelial cells. *BMC Cancer* **1**, 19.
- [104] Flowers LO, Subramaniam PS, and Johnson HM (2005). A SOCS-1 peptide mimetic inhibits both constitutive and IL-6 induced activation of STAT3 in prostate cancer cells. *Oncogene* **24**, 2114–2120.
- [105] Li H, Ahonen TJ, Alanen K, Xie J, LeBaron MJ, Pretlow TG, Ealley EL, Zhang Y, Nurmi M, Singh B, et al. (2004). Activation of signal transducer and activator of transcription 5 in human prostate cancer is associated with high histological grade. *Cancer Res* **64**, 4774–4782.
- [106] Weiss-Messer E, Merom O, Adi A, Karry R, Bidosee M, Ber R, Kaploun A, Stein A, and Barkey RJ (2004). Growth hormone (GH) receptors in prostate cancer: gene expression in human tissues and cell lines and characterization, GH signaling and androgen receptor regulation in LNCaP cells. *Mol Cell Endocrinol* **220**, 109–123.
- [107] Bowen C, Birrer M, and Gelmann EP (2002). Retinoblastoma protein-mediated apoptosis after gamma-irradiation. *J Biol Chem* **277**, 44969–44979.
- [108] Macoska JA, Xu J, Ziemnicka D, Schwab TS, Rubin MA, and Kotula L (2001). Loss of expression of human spectrin src homology domain binding protein 1 is associated with 10p loss in human prostatic adenocarcinoma. *Neoplasia* **3**, 99–104.
- [109] Kubler HR, van Randenborgh H, Treiber U, Wutzler S, Battistel C, Lehmer A, Wagenpfeil S, Hartung R, and Paul R (2005). *In vitro*

- cytotoxic effects of imatinib in combination with anticancer drugs in human prostate cancer cell lines. *Prostate* **63**, 385–394.
- [110] Uehara H, Kim SJ, Karashima T, Shepherd DL, Fan D, Tsan R, Killian JJ, Logothetis C, Mathew P, and Fidler IJ (2003). Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. *J Natl Cancer Inst* **95**, 458–470.
- [111] Kim SJ, Uehara H, Yazici S, Langley RR, He J, Tsan R, Fan D, Killian JJ, and Fidler IJ (2004). Simultaneous blockade of platelet-derived growth factor-receptor and epidermal growth factor-receptor signaling and systemic administration of paclitaxel as therapy for human prostate cancer metastasis in bone of nude mice. *Cancer Res* **64**, 4201–4208.
- [112] Kim SJ, Uehara H, Yazici S, He J, Langley RR, Mathew P, Fan D, and Fidler IJ (2005). Modulation of bone microenvironment with zoledronate enhances the therapeutic effects of STI571 and paclitaxel against experimental bone metastasis of human prostate cancer. *Cancer Res* **65**, 3707–3715.
- [113] Corcoran NM and Costello AJ (2005). Combined low-dose imatinib mesylate and paclitaxel lack synergy in an experimental model of extraosseous hormone-refractory prostate cancer. *BJU Int* **96**, 640–646.
- [114] Mathew P, Thall PF, Jones D, Perez C, Bucana C, Troncoso P, Kim SJ, Fidler IJ, and Logothetis C (2004). Platelet-derived growth factor receptor inhibitor imatinib mesylate and docetaxel: a modular phase I trial in androgen-independent prostate cancer. *J Clin Oncol* **22**, 3323–3329.
- [115] Tiffany NM, Wersinger EM, Garzotto M, and Beer TM (2004). Imatinib mesylate and zoledronic acid in androgen-independent prostate cancer. *Urology* **63**, 934–939.
- [116] Rao K, Goodin S, Levitt MJ, Dave N, Shih WJ, Lin Y, Capanna T, Doyle-Lindrud S, Juvidian P, and DiPaola RS (2005). A phase II trial of imatinib mesylate in patients with prostate specific antigen progression after local therapy for prostate cancer. *Prostate* **62**, 115–122.
- [117] Bjorge JD, O'Connor TJ, and Fujita DJ (1996). Activation of human pp60c-src. *Biochem Cell Biol* **74**, 477–484.
- [118] Tamagnone L, Lahtinen I, Mustonen T, Virtaneva K, Francis F, Muscatelli F, Alitalo R, Smith CI, Larsson C, and Alitalo K (1994). *BMX*, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TKX family located in chromosome Xp22.2. *Oncogene* **9**, 3683–3688.
- [119] Qiu Y, Robinson D, Pretlow TG, and Kung HJ (1998). Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. *Proc Natl Acad Sci* **95**, 3644–3649.
- [120] Xue LY, Qiu Y, He J, Kung HJ, and Oleinick NL (1999). Etk/Bmx, a PH-domain containing tyrosine kinase, protects prostate cancer cells from apoptosis induced by photodynamic therapy or thapsigargin. *Oncogene* **18**, 3391–3398.
- [121] Jiang T, Guo Z, Dai B, Kang M, Ann DK, Kung HJ, and Qiu Y (2004). Bi-directional regulation between tyrosine kinase Etk/BMX and tumor suppressor p53 in response to DNA damage. *J Biol Chem* **279**, 50181–50189.
- [122] Wu YM, Huang CL, Kung HJ, and Huang CY (2001). Proteolytic activation of ETK/Bmx tyrosine kinase by caspases. *J Biol Chem* **276**, 17672–17678.
- [123] Kim O, Jiang T, Xie Y, Guo Z, Chen H, and Qiu Y (2004). Synergism of cytoplasmic kinases in IL6-induced ligand-independent activation of androgen receptor in prostate cancer cells. *Oncogene* **23**, 1838–1844.
- [124] Xie Y, Xu K, Dai B, Guo Z, Jiang T, Chen H, and Qiu Y (2006). The 44 kDa Pim-1 kinase directly interacts with tyrosine kinase Etk/BMX and protects human prostate cancer cells from apoptosis induced by chemotherapeutic drugs. *Oncogene* **25**, 70–78.
- [125] Wang Y, Yu Q, Cho AH, Rondeau G, Welsh J, Adamson E, Mercola D, and McClelland M (2005). Survey of differentially methylated promoters in prostate cancer cell lines. *Neoplasia* **7**, 748–760.
- [126] Hoehn GT, Stokland T, Amin S, Ramirez M, Hawkins AL, Griffin CA, Small D, and Civin CI (1996). TnK1: a novel intracellular tyrosine kinase gene isolated from human umbilical cord blood CD34<sup>+</sup>/Lin<sup>−</sup>/CD38<sup>−</sup> stem/progenitor cells. *Oncogene* **12**, 903–913.
- [127] Saitou N and Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

# Biologic agents as adjunctive therapy for prostate cancer: a rationale for use with androgen deprivation

Eric C Nelson, Angelo J Cambio, Joy C Yang, Primo N Lara Jr and Christopher P Evans\*

## SUMMARY

The prevalence of prostate cancer emphasizes the need for improved therapeutic options, particularly for metastatic disease. Current treatment includes medical or surgical castration, which initially induces apoptosis of prostate cancer cells, but ultimately an androgen-independent subpopulation emerges. In addition to a transient therapeutic effect, androgen-deprivation therapy (ADT) can initiate biochemical events that may contribute to the development of and progression to an androgen-independent state. This transition involves multiple signal transduction pathways that are accompanied by many biochemical changes resulting from ADT. These molecular events themselves are therapeutic targets and serve as a rationale for adjunctive treatment at the time of ADT.

**KEYWORDS** androgen-deprivation, androgen-independent, castration, hormone refractory prostate cancer, prostate cancer

## REVIEW CRITERIA

A PubMed search of the English language literature from 1990 to 2006 for pertinent articles was conducted using the MeSH term "Prostate neoplasms" in conjunction with terms such as "biochemistry," "mechanism," "androgen independent," "metastatic," and "treatment." The bibliographies of retrieved articles were scrutinized for additional articles. Identified concepts, biochemical factors, and treatment strategies were searched again using MeSH terms. Particularly relevant articles were input into Web of Science to retrieve the latest citations and articles.

EC Nelson is a Urology Research Fellow, JC Yang is an Assistant Research Biologist, PN Lara Jr is Professor of Medical Oncology and CP Evans is Associate Professor and Chair of the Urology Department, University of California, Davis, CA. AJ Cambio is a Urology Resident at the University of Rochester, Rochester, NY, USA.

## Correspondence

\*Department of Urology, University of California, Davis Medical Center, Suite 3500, 4860 Y Street, Sacramento, CA 95817, USA  
christopher.evans@ucdmc.ucdavis.edu

Received 14 September 2006 Accepted 15 November 2006

www.nature.com/clinicalpractice  
doi:10.1038/ncpuro0700

## INTRODUCTION

Prostate cancer remains the most common noncutaneous malignancy in the US and is predicted to be the third leading cause of cancer mortality in 2006.<sup>1</sup> Currently, androgen-deprivation therapy (ADT) is the mainstay of therapy for metastatic disease and is principally palliative in nature. ADT removes androgen stimulation, initially inducing apoptotic involution of prostatic tissue. The disease, however, eventually progresses to an androgen-independent state, which is associated with a life-expectancy of approximately 15–20 months.<sup>2</sup> Despite research efforts and recent advances using chemotherapeutic agents, minimal progress has been made in the treatment of advanced prostate cancer in the last 50 years, and the life expectancy of patients with metastatic disease has changed very little. ADT, while extending length and quality of life for many patients, induces biological changes in prostate cancer cells that promote an androgen-independent state.

When prostate cancer becomes androgen independent, methods of growth stimulation other than those mediated by androgens dominate, allowing progression of the disease. The androgen receptor (AR) continues to be expressed and even amplified in androgen-independent prostate cancer, and androgen responsive genes are re-expressed when cancer progresses to the androgen-independent state.<sup>3</sup> As such, much research has focused on the AR in order to understand the method of continued AR-stimulated transcription in the absence of a natural ligand. Many possible pathways have been explored. AR amplification, mutation, and hypersensitivity might allow continued signaling with low androgen levels. Alternate pathways might activate the AR by phosphorylation in an androgen-independent manner. Finally, pathways that regulate apoptosis and cell proliferation, but without direct AR effects, might oppose the effects of ADT. The current understanding of possible mechanisms involved in the development of

androgen-independent prostate cancer has been the subject of two reviews.<sup>4,5</sup>

This paper focuses on the mechanisms activated or upregulated with ADT that currently show potential as targets for treatment for advanced prostate cancer and have a theoretical rationale for castration-adjunctive therapy. We summarize selected pathways and therapies, dividing our discussion into AR-dependent and AR-independent categories (Figure 1).

## ANDROGEN-RECEPTOR-DEPENDENT PATHWAYS

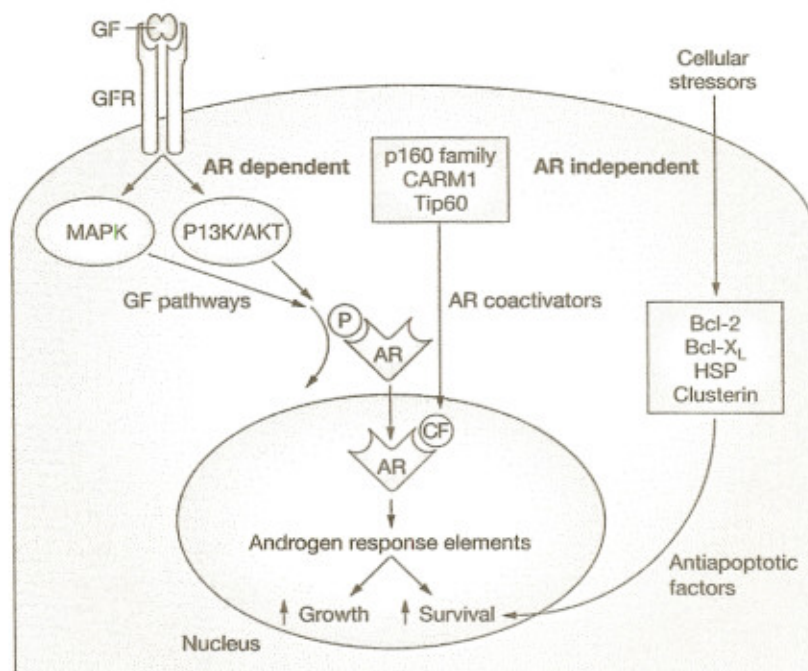
### Androgen-receptor cofactors

Activity of the AR is modulated by many cofactors that can increase or decrease its transcriptional activity.<sup>6</sup> Coactivators permit receptor activation with the very low levels of androgen typically seen in the castrate setting, or facilitate greater AR activity with aberrant stimulation from a number of pathways (outlined below).<sup>4</sup> Several families of cofactors have been shown to change expression levels in response to ADT (Table 1), indicating a mechanism for the development of androgen-independent disease. Feldman and Feldman<sup>4</sup> speculate that loss of corepressors might be another mechanism for AR activation. We are not aware of any current therapy aimed at increasing corepressors or decreasing coactivators of the AR. The consistency of the castration-induced changes and strength of the effects of these proteins in preclinical studies, however, indicate that they might be targets for novel therapies in the future.<sup>7</sup>

### Growth factors

Growth factors are the major paracrine signaling mechanism used by the prostatic stroma and neuroendocrine cells. In healthy prostate glands, growth factors regulate growth and cellular maturation. In the setting of androgen-independent tumors, growth factors influence an alternate pathway that activates AR-mediated signaling. In general, growth factors and neuropeptides bind cell membrane receptors, which either polymerize to activate intracellular tyrosine kinase domains, or activate associated G-proteins that in turn activate nonreceptor tyrosine kinases (Figure 2).

One of the best characterized growth factors is the epidermal growth factor (EGF) family including transforming growth factor  $\alpha$  (TGF $\alpha$ ), EGF, amphiregulin and others. These bind the



**Figure 1** Three possible pathways leading to androgen-independent prostate cancer. Growth factor signaling cascades may activate the AR by phosphorylation in the absence of ligand. Coactivators modify transcriptional activity of the AR. When activated, the AR enters the nucleus, binds to androgen response elements, and transcribes specific genes promoting survival and proliferation. Antiapoptotic factors increase in response to cellular stressors, also promoting survival. Abbreviations: AR, androgen receptor; CARM1, coactivator-associated arginine methyltransferase 1; CF, cofactor; GF, growth factor; GFR, growth factor receptor; HSP, heat-shock protein; P, phosphate group; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; Tip60, Tat interacting protein 60 kDa.

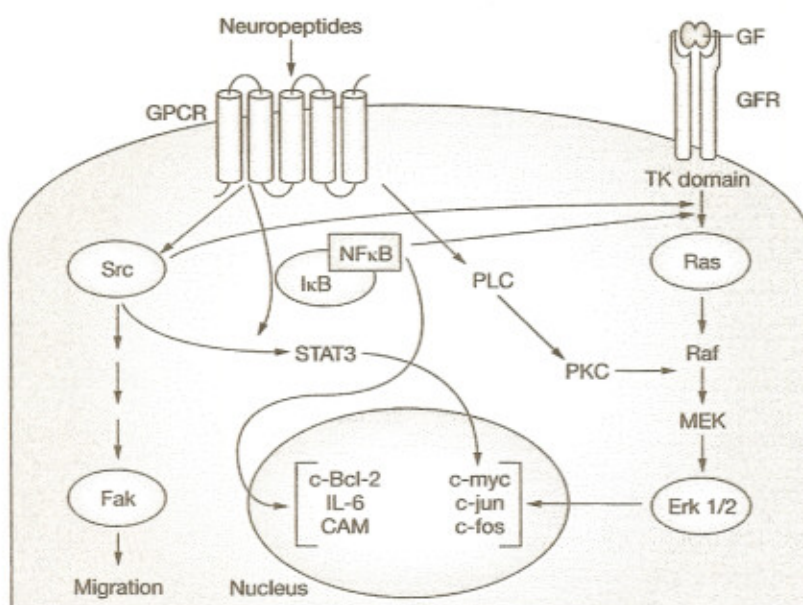
EGF receptor (EGFR), activating the intracellular tyrosine kinase domain with subsequent effects mediated by multiple pathways. Two of the most important involve mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K). Both of these pathways can activate the AR by phosphorylation independently of a natural ligand, in addition to other effects that promote cell proliferation and oppose apoptosis in an androgen-independent manner. Consistent with this mechanism, activation of MAPK was shown to be increased following ADT.<sup>8</sup>

Expression of the EGFR and its subtype HER2/neu in prostate cancer is the subject of controversy. Lorenzo *et al.*<sup>9</sup> reviewed 14 studies, which variously showed expression of HER2/neu to be present in 0–100% of prostate tumors. There is, however, greater uniformity in evidence supporting HER2/neu upregulation by ADT.

**Table 1** Castration-induced changes in androgen receptor cofactors possibly involved in androgen receptor-dependent prostate cancer progression.

| Factor                   | CaP related physiology  | Castration related changes                      | Potential clinical role                                    | Selected references |
|--------------------------|---|---|--|---------------------|
| ARA70                    | AR coactivator  | Initial decrease; increased with AI progression | Future: blockade may decrease AI AR signaling              | 54                  |
| CARM1 <sup>a</sup>       | AR coactivator <sup>a</sup>   | Increased <sup>a</sup>                          | Future target <sup>a</sup>                                 | 55                  |
| p160 family <sup>a</sup> | Family of AR coactivators including Src-1, TIF-2 and RAC-3 <sup>a</sup> | Increased <sup>a</sup>                          | Future: blockade may decrease AI AR signaling <sup>a</sup> | 7                   |
| p300 (CBP)               | AR coactivator  | Unknown   | Future: blockade may decrease AI AR signaling              | 56                  |
| Tip60 <sup>a</sup>       | AR coactivator <sup>a</sup>   | Increased <sup>a</sup>                          | Future: blockade may decrease AI AR signaling <sup>a</sup> | 57                  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: AI, androgen independent; AR, androgen receptor; ARA70, androgen receptor associated protein 70; CaP, prostate cancer; CARM1, coactivator-associated arginine methyltransferase 1; CBP, CREB binding protein; RAC-3, nuclear receptor coactivator 3; Tip60, Tat interacting protein 60 kDa; TIF-2, transcription intermediary factor 2.



**Figure 2** Comparison of growth factor and neuropeptide signaling pathways emphasizing cross-talk between them. Growth factors activate receptors with intracellular TK domains, subsequently activating a signaling cascade through Ras leading to modification of transcription. Neuropeptides activate GPCR, activating Src and NF-κB by separation from its binding protein IκB. Both of these may activate growth factor receptor TK domains as well as affecting transcription of various factors. The GPCR may also activate other signaling cascades through Src and PLC/PKC. Abbreviations: CAM, cell adhesion molecule; GF, growth factor; GFR, growth factor receptor; GPCR, G-protein coupled receptor; IκB, inhibitor of κB; IL-6, interleukin 6; NF-κB, nuclear factor κB; PKC, protein kinase C; PLC, phospholipase C; TK, tyrosine kinase.

Three of four studies reported that there were significant increases in this receptor following administration of ADT, and the study that did not demonstrate this effect reported a significant increase in EGFR overall compared to pre-ADT levels. Phase II trials of the monoclonal antibody (mAb) against HER2/neu, trastuzumab, have, however, been limited due to low levels of expression of the receptor in the screened patients. Screening produced generally disappointing results in the few patients tested.<sup>10,11</sup> Other trials of mAbs against the extracellular or intracellular domains of the EGFR are ongoing.<sup>12,13</sup> Other therapeutic options that target the EGFR include small-molecule inhibitors of the intracellular tyrosine kinase domain, such as gefitinib and erlotinib. Unfortunately, trials using these drugs as monotherapies have not demonstrated a clear benefit to patients.<sup>14</sup>

The insulin-like growth factor (IGF) pathway functions in a similar way to the pathways outlined earlier. In contrast to EGF, no consistent changes in IGF or its receptors are noted in men who have undergone castration,<sup>5</sup> but two patients with advanced disease did show increased IGF-1 receptor levels.<sup>15</sup> Of more interest is the finding that the levels of several IGF binding proteins (IGFBP) change in response to ADT; IGFBP2 and IGFBP5 both increase acutely and IGFBP3 decreases within weeks of initiation of ADT.<sup>16–18</sup> All of these changes might increase the effect of IGF signaling through the PI3K pathway.

Many other growth factors, neuropeptides and cytokines have been shown to activate signaling pathways in a similar manner to IGF and EGF, resulting in activation of the AR (Tables 2–5). The downregulation of neutral endopeptidase at castration is thought to provide an important source for increasing levels of growth factors and neuropeptides, as the enzyme is the primary means of degrading these cellular signals.<sup>19</sup> Almost all of the specific growth factors and receptors have antagonists that are undergoing early clinical trials. These were recently reviewed by van der Poel.<sup>12</sup>

The multiplicity of growth factors and neuropeptides leads to significant cross-talk between the cell signaling pathways (Figure 2). The many pathways lead to the same effectors, which might make downstream targets, that impact on multiple pathways, more likely to exhibit significant inhibitory effects. One such target is the Src family kinases, which are

**Table 2** Castration-induced changes in growth factors possibly involved in androgen receptor-dependent prostate cancer progression.

| Factor                        | CaP related physiology   | Castration related changes                                  | Potential clinical role   | Selected references |
|-------------------------------|--|---|---|---------------------|
| EGF family                    | Ligand for receptor tyrosine kinases initiating multiple signaling cascades              | Some are increased  | See EGF receptor blockers   | 58                  |
| FGF family <sup>a</sup>       | Binds receptor with generally trophic effects <sup>a</sup>                               | FGF-8 increased <sup>a</sup>                                | TNP-470 inhibits binding, no effect in early trials <sup>a</sup>                      | 58, 59              |
| HGF <sup>a</sup>              | Coactivator of AR <sup>a</sup>   | Increased <sup>a</sup>                                      | Future inhibitor may decrease AR effects in AI CaP <sup>a</sup>                       | 60                  |
| IGF-1 <sup>a</sup>            | Ligand for receptor tyrosine kinases initiating multiple signaling cascades <sup>a</sup> | No change <sup>a</sup>                                      | Future inhibitor may decrease AR effects in AI CaP <sup>a</sup>                       | 5, 58               |
| IGFBP-2, IGFBP-5 <sup>a</sup> | Increases IGF signal transduction <sup>a</sup>   | Increased <sup>a</sup>                                      | ASOs show activity <sup>a</sup>   | 16, 17, 61          |
| IGFBP-3 <sup>a</sup>          | Decreases IGF signal transduction <sup>a</sup>   | Decreased <sup>a</sup>                                      | Prognostic indicator <sup>a</sup>   | 18                  |
| TGF- $\beta$ 1 <sup>a</sup>   | Ligand for receptor tyrosine kinases initiating multiple signaling cascades <sup>a</sup> | Increased in some <sup>a</sup>                              | Prognostic factor <sup>a</sup>  | 35                  |
| VEGF <sup>a</sup>             | Angiogenesis and cell motility <sup>a</sup>  | Initial decrease; NE cells continue to produce <sup>a</sup> | mAb to VEGF (bevacizumab) specifically inhibits tyrosine kinase activity <sup>a</sup> | 12, 36, 62, 63      |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: AI, androgen independent; AR, androgen receptor; ASO, antisense oligonucleotide; CaP, prostate cancer; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IGFBP, IGF binding protein; mAb, monoclonal antibody; NE, neuroendocrine; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

**Table 3** Castration-induced changes in receptors possibly involved in androgen receptor-dependent prostate cancer progression.

| Factor  | CaP related physiology   | Castration related changes                 | Potential clinical role  | Selected references |
|---|--|--|--|---------------------|
| EGFR <sup>a</sup>   | Receptor with intracellular tyrosine kinase domain activating multiple pathways <sup>a</sup> | Increased <sup>a</sup>                     | Multiple mAb and tyrosine kinase inhibitors <sup>a</sup>                                     | 9, 12, 64           |
| ErbB2/HER-2/neu   | Receptor with intracellular tyrosine kinase domain activating multiple pathways              | ADT selects for HER-2/neu expressing cells | Multiple mAb and tyrosine kinase inhibitors  | 9, 12               |
| FGFR-2 <sup>a</sup>   | Binds ligand with effects on angiogenesis and wound healing <sup>a</sup>                     | Decreased <sup>a</sup>                     | NR   | 65                  |
| IGF-1R <sup>a</sup>   | Binds ligand initiating multiple signaling cascades <sup>a</sup>                             | Increased in 2 patients <sup>a</sup>       | Small molecule inhibitors show some activity in other cancers <sup>a</sup>                   | 15                  |
| TGF- $\beta$ 1R 1 <sup>a</sup> and TGF- $\beta$ 1R 2 <sup>a</sup>     | Binds ligand with complex cell signaling effects <sup>a</sup>                                | Increased in some cancers <sup>a</sup>     | Prognostic factor <sup>a</sup>   | 35                  |
| Serotonin receptor 2B, <sup>a</sup> serotonin receptor 4 <sup>a</sup> | Active in autocrine and paracrine signaling supporting AI growth <sup>a</sup>                | Receptor 4 increased <sup>a</sup>          | Serotonin receptor antagonists inhibit CaP cell growth; AI cells more sensitive <sup>a</sup> | 66                  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; CaP, prostate cancer; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; IGF-1R, insulin-like growth factor receptor 1; mAb, monoclonal antibody; NR, not reported; TGF $\beta$ 1R, transforming growth factor receptor- $\beta$ 1.

**Table 4** Castration-induced changes in neuropeptides possibly involved in androgen receptor-independent prostate cancer progression.

| Factor                    | CaP related physiology  | Castration related changes   | Potential clinical role  | Selected references |
|---------------------------|---|--|--|---------------------|
| Calcitonin                | Angiogenesis, invasiveness stimulation through PKA, AKT and others                  | Produced by NE cells unaffected by ADT   | Blockade may allow apoptosis in response to chemotherapy                                 | 68, 76              |
| Endothelin-1              | Angiogenesis, osteoblastic lesion formation, neuropeptide for multiple receptors    | Increased in CaP in general; endothelin receptors increased by ADT             | Endothelin-A receptor antagonist, atrasentan, in phase II/III studies <sup>a</sup>       | 12, 21, 38, 69      |
| ProGRP <sup>a</sup>       | Proneuropeptide <sup>a</sup>  | Follows level of NED <sup>a</sup>  | Marker for NED <sup>a</sup>  | 70                  |
| GRP/ bombesin             | Stimulates AI growth; multiple effects through NFκB                                 | Follows level of NED   | Marker for NED; prognostic utility   | 20, 67, 68, 70, 71  |
| NEP (CD10) <sup>a</sup>   | Cleaves many neuropeptides controlling extracellular concentrations <sup>a</sup>    | Decreased <sup>a</sup>   | Increasing NEP expression through gene constructs possible future treatment <sup>a</sup> | 19                  |
| Neurotensin               | Neuropeptide stimulates androgen independent growth                                 | Increased in NE cells  | Marker for NED   | 20, 21, 72          |
| PTHrP                     | Multiple bioactive proteins from initial transcript; generally trophic neuropeptide | Increased  | Anti-PTHrP mAb inhibits hypercalcemia of malignancy                                      | 67, 72              |
| Serotonin <sup>a</sup>    | Binds multiple receptors supporting AI growth <sup>a</sup>                          | Increased <sup>a</sup>   | Serotonin antagonists <sup>a</sup>   | 66, 67, 73          |
| Somatostatin <sup>a</sup> | Inhibits angiogenesis, proliferation, stimulates apoptosis <sup>a</sup>             | Receptors are upregulated in CaP in general; produced by NE cells <sup>a</sup> | Somatostatin analogs <sup>a</sup>  | 73                  |
| VIP                       | Activates ERK 1/2, PI3K and other pathways supporting AI growth                     | Unknown  | NR   | 74                  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; AI, androgen independent; CaP, prostate cancer; ERK, extracellular-signal-regulated protein kinase; GRP, gastrin releasing peptide; mAb, monoclonal antibody; NE, neuroendocrine; NED, neuroendocrine differentiation; NEP, neutral endopeptidase; NFκB, nuclear factor κB; NR, not reported; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PTHrP, parathyroid hormone related protein; VIP, vasoactive intestinal peptide.

nonreceptor tyrosine kinases that participate in a variety of intracellular pathways stimulated by neuropeptides.<sup>20</sup> In addition, Src kinases can activate STAT3, a transcription factor that subsequently activates cyclin D1, vascular endothelial growth factor (VEGF), and c-myc.<sup>21</sup> The activities represented by these pathways are clearly involved in the progression of androgen-independent prostate cancer. Two selective non-receptor tyrosine kinase inhibitors, MBS-354825 (dasatinib) and AZD0530, are both undergoing phase II investigation sponsored by the National Cancer Institute for use in patients with androgen-independent prostate cancer.<sup>22,23</sup> Inhibitory molecules, such as the nonreceptor tyrosine kinases, have shown therapeutic promise *in vitro*<sup>24</sup> and might prove

to be good treatment options for men with androgen-independent prostate cancer.

## ANDROGEN-RECEPTOR-INDEPENDENT PATHWAYS

### Antiapoptotic pathways

Multiple pathways lead to apoptosis and several cellular proteins involved in apoptosis show changes following castration (Table 6). The Bcl family of proteins includes both proapoptotic and antiapoptotic members, the balance of which is critical for maintaining cellular homeostasis. The antiapoptotic Bcl-2 is consistently upregulated by castration and probably has an important role in allowing androgen-independent progression of prostate cancer.<sup>25-27</sup> The antisense oligonucleotide (ASO) against

**Table 5** Castration-induced changes in interleukins possibly involved in androgen receptor-independent prostate cancer progression.

| Factor                | CaP related physiology  | Castration related changes                             | Potential clinical role  | Selected references |
|-----------------------|---|--|--|---------------------|
| IL-1 $\alpha/\beta^a$ | Increase bone resorption <sup>a</sup>   | Serum levels decreased <sup>a</sup>                    | NR   | 75                  |
| IL-3 <sup>a</sup>     | Increase bone resorption <sup>a</sup>   | Serum levels unchanged <sup>a</sup>                    | NR   | 75                  |
| IL-4 <sup>a</sup>     | Modulate inflammation <sup>a</sup>  | Serum levels increase when CaP becomes AI <sup>a</sup> | Marker <sup>a</sup>  | 76                  |
| IL-6 <sup>a</sup>     | Activates multiple signaling cascades supporting growth, differentiation and cell survival <sup>a</sup> | Serum levels increase when CaP becomes AI <sup>a</sup> | Anti-IL-6 mAbs in early development <sup>a</sup>                           | 73, 76              |
| IL-8                  | Angiogenesis, metastasis, AI growth through Src and FAK   | May be increased if TGF- $\beta$ is increased          | NR   | 77                  |
| IL-10 <sup>a</sup>    | Modulates inflammation <sup>a</sup>   | Serum levels increase when CaP becomes AI <sup>a</sup> | Marker <sup>a</sup>  | 76                  |
| M-CSF <sup>a</sup>    | Increase bone resorption <sup>a</sup>   | Unchanged <sup>a</sup>                                 | Future: blockade may delay metastasis <sup>a</sup>                         | 75                  |
| Oncostatin M          | Related to IL-6; activates AR   | Decreased  | Future: blocking pathway might reduce AI-AR stimulation                    | 61                  |
| TNF- $\alpha^a$       | Inhibits apoptosis through NF $\kappa$ B <sup>a</sup>   | Unchanged <sup>a</sup>                                 | Future: blockade may promote apoptosis and inhibit metastasis <sup>a</sup> | 75, 78              |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: AI, androgen independent; AR, androgen receptor; CaP, prostate cancer; FAK, focal adhesion kinase; IL, interleukin; M-CSF, macrophage colony stimulating factor; mAb, monoclonal antibody; NF $\kappa$ B, nuclear factor  $\kappa$ B; NR, not reported; TGF, transforming growth factor; TNF, tumor necrosis factor.

**Table 6** Castration-induced changes in apoptosis regulators possibly involved in androgen receptor-independent prostate cancer progression.

| Factor                                  | CaP related physiology   | Castration related changes                | Potential clinical role  | Selected references |
|---|--|---|--|---------------------|
| Bcl-2 <sup>a</sup>                      | Family of factors both proapoptotic and antiapoptotic; balance determines cell survival <sup>a</sup> | Increased <sup>a</sup>                    | ASO oblimersen sodium is undergoing trials <sup>a</sup>  | 25–27, 64           |
| Clusterin <sup>a</sup>                  | Nuclear chaperone inhibits apoptosis <sup>a</sup>  | Increased <sup>a</sup>                    | ASOs show promise in early trials <sup>a</sup>   | 31, 32              |
| HSP27, HSP60, HSP70, HSP90 <sup>a</sup> | Nuclear chaperones bind denatured proteins; prevent apoptosis <sup>a</sup>                           | Most increased <sup>a</sup>               | ASO and siRNA to HSP27 in early trials; geldanamycin and other ansamycins inhibit HSP90 <sup>a</sup> | 30, 61              |
| IAPs                                    | Apoptotic inhibitor  | Survivin and others increased in NE cells | ASOs have shown promise; must target multiple IAPs   | 79                  |
| p53 <sup>a</sup>                        | Cell cycle regulator; proapoptotic <sup>a</sup>  | Increased <sup>a</sup>                    | NR   | 34, 64, 80          |
| p21 (WAF1, CIP1) <sup>a</sup>           | Effector for p53; halts cell cycle in G1 <sup>a</sup>  | Increased <sup>a</sup>                    | Prognostic indicator <sup>a</sup>  | 34                  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: ASO, antisense oligonucleotide; CaP, prostate cancer; HSP, heat shock protein; IAP, inhibitor-of-apoptosis protein; NE, neuroendocrine; NR, not reported; siRNA, small interfering RNA.

Bcl-2 has shown activity, both *in vitro* and *in vivo*, delaying the progression to androgen-independent disease.<sup>28</sup> Recent phase II results in androgen-independent prostate cancer showed tolerability in combination with docetaxel and an impressive 80% response rate in the subset of patients achieving high serum concentrations of the oligonucleotide.<sup>29</sup>

Another cellular pathway leading to apoptosis is induced by the accumulation of denatured proteins in the cell. Cellular stress leads to this condition and increasing numbers of dysfunctional proteins may precipitate, signaling caspase activation. The term 'heat shock protein' (HSP) encompasses a large family of related proteins that function as 'chaperones' to stabilize the

**Table 7** Castration-induced changes in cell wall factors possibly involved in androgen receptor-independent prostate cancer progression.

| Factor                           | CaP related physiology  | Castration related changes                     | Potential clinical role   | Selected references |
|----------------------------------|---|--|---|---------------------|
| Caveolin                         | Component of plasma membrane pits involved in cell signaling                                      | Decreased                                      | NR  | 81                  |
| FAK                              | Cell motility/metastasis; signaling pathways  | Possibly increased if NE GF pathways increased | Genistein alters FAK activity, may reduce metastasis                              | 46, 77              |
| Fibronectin                      | Cell adhesion through interaction with integrin   | Increased 60× <i>in vitro</i>                  | Future: blocking upregulation to decrease metastatic potential                    | 43                  |
| ILK                              | Protein kinase linking cell adhesion to signaling; negatively regulated by PTEN                   | None   | Future: inhibit in tumors with PTEN mutation                                      | 82                  |
| MMP-2, MMP-9                     | Cleaves basement membrane constituents; possible role in invasion/metastasis                      | Inconsistent                                   | Many MMP inhibitors may have clinical utility in combination; prognostic utility  | 83                  |
| Mucin                            | Family of glycoproteins modulating cell adhesion  | Most are increased                             | Vaccines targeting MUC-1 peptide <sup>a</sup>                                     | 44, 45              |
| Syndecan-1 (CD-138) <sup>a</sup> | Binds matrix proteins and growth factors; involved with adhesion and differentiation <sup>a</sup> | Increased <sup>a</sup>                         | Possible role in immunotherapy <sup>a</sup>                                       | 64, 84              |
| TIMP-1, TIMP-2                   | Inhibits MMPs   | Inconsistent; opposite MMP                     | Analogues could decrease MMP expression; regulation of endogenous levels possible | 85, 86              |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: CaP, prostate cancer; FAK, focal adhesion kinase; GF, growth factor; ILK, integrin-linked kinase; MMP, matrix metalloproteinase; MUC-1, urinary mucin 1; NE, neuroendocrine; NR, not reported; PTEN, phosphatase and tensin homolog; TIMP, tissue inhibitor of matrix metalloproteinase.

denatured proteins that result from cellular stress. The cell thereby becomes more resistant to apoptosis induced by multiple factors such as heat, radiation, and toxic drugs. As would be expected, many of the HSPs change expression in response to the stress of castration (Table 6). HSP27 has been extensively studied and might represent another important pathway of resistance to apoptosis in the androgen-independent state. In addition to its functions as a chaperone, HSP27 also directly interferes with caspase activation, modulates cellular oxidative stress, and stabilizes the cytoskeleton. The upregulation of HSP27 can be targeted by ASOs or small interfering RNA, and both of these therapies are entering phase I/II trials.<sup>30</sup>

Clusterin is a nuclear protein that also functions as a chaperone. One isoform of clusterin shows greater potency than even heat shock proteins to 'stabilize' denatured proteins. Levels of clusterin increase significantly following castration and might be an important signal inhibiting apoptosis.<sup>31,32</sup> An ASO against the clusterin gene successfully decreases expression, causing an increase in apoptosis in cells exposed to multiple chemotherapeutic drugs, radiation,

and oxidative stress. A phase I trial showed the clusterin ASO OGX-011 to have acceptable toxicity at effective plasma concentrations.<sup>33</sup> Phase II trials are underway to further define the role of ASOs in prostate cancer therapy.<sup>30</sup>

The antiapoptotic changes discussed above can be counteracted by the p53 protein, which inhibits the cell cycle when DNA damage has occurred. After the dividing cell is held in the G1 stage, apoptosis is induced by a mechanism (not yet fully understood) involving both the p53 protein and its effector p21. Both these proteins are upregulated by castration. It has, however, been suggested that one or both might be mutated and nonfunctional.<sup>34</sup> This would upset the balance of proapoptotic and antiapoptotic signals within the cell, allowing resistance to ADT to develop and thereby facilitating the emergence of androgen-independent prostate cancer.

#### Miscellaneous pathways

The intricate pathways of cellular homeostasis show wide-ranging effects following castration. The brief discussion of the following three pathways highlights their potential importance to castration-adjunctive therapy.

**Table 8** Castration-induced changes in signal transduction factors possibly involved in androgen receptor-independent prostate cancer progression.

| Factor            | CaP related physiology   | Castration related changes   | Potential clinical role  | Selected references |
|-------------------|--|--|--|---------------------|
| AKT <sup>a</sup>  | Antiapoptotic <sup>a</sup>   | Possible increase if NE cells switch to GF pathways <sup>a</sup>       | Testing of specific inhibitors ongoing <sup>a</sup>                                | 4, 83               |
| DHT <sup>a</sup>  | Binds AR activating transcriptional activity <sup>a</sup>                | Decreased <sup>a</sup>   | ADT standard of care <sup>a</sup>  | 87                  |
| MAPK <sup>a</sup> | Protein kinase; activates multiple pathways <sup>a</sup>                 | Increased in 2 patients, associated with advanced disease <sup>a</sup> | Future: blockade may decrease AI AR stimulation <sup>a</sup>                       | 8                   |
| Nkx3.1            | Protein-protein interactions modify cell signaling; transcription factor | Initial decrease; increased with AI progression                        | Future: replacement may modify effects of PTEN mutation                            | 88                  |
| PI3K              | Protein kinase; activates multiple pathways                              | Possible increase if GF pathways increase activity                     | Wortmannin derivatives and peptidomimetics may be helpful in blocking this pathway | 89                  |
| PKC <sup>a</sup>  | Protein kinase; activates multiple pathways <sup>a</sup>                 | Variable <sup>a</sup>  | ASO in early trials; prognostic factor <sup>a</sup>                                | 90                  |
| PTEN <sup>a</sup> | Proapoptotic through inactivation of AKT pathway <sup>a</sup>            | Lost to mutation in most cancers <sup>a</sup>                          | Competitive inhibitors under development; see AKT                                  | 4                   |
| Src               | Involved in multiple pathways activating AR                              | Increased if GF pathways increase activity                             | Dasatinib, AZD0530 inhibits Src family kinases                                     | 21, 24, 77, 91, 92  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; AI, androgen independent; AR, androgen receptor; ASO, antisense oligonucleotide; CaP, prostate cancer; DHT, dihydrotestosterone; GF, growth factor; MAPK, mitogen-activated protein kinase; NE, neuroendocrine; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PTEN, phosphatase and tensin homolog.

Angiogenesis is necessary for tumor growth, and some of the factors known to be upregulated in androgen-independent prostate cancer stimulate angiogenesis (Table 2). For example, TGF $\beta$  is upregulated following castration,<sup>35</sup> and VEGF is produced by androgen-independent neuroendocrine cells, which are relatively unaffected by ADT.<sup>36</sup> Furthermore, VEGF levels are an independent prognostic factor in androgen-independent prostate cancer.<sup>37</sup> Broad spectrum antiangiogenic drugs such as suramin analogs and thalidomide have shown activity, singly and in combination, in early trials.<sup>38</sup> Targeted treatment with the mAb against VEGF, bevacizumab, showed some activity in combination with chemotherapy.<sup>39</sup> Ongoing trial results are awaited.

Multiple factors regulating cellular adhesion change expression level in response to ADT (Tables 7 and 8), and those that do not change may still be attractive targets for treatment to inhibit invasion or metastasis. Antimetastatic therapies might have the added advantage of modifying cellular signaling in addition to their intended cellular adhesion effects. For example, fibronectin interacts with integrins to affect cellular adhesion. These interactions also activate intracellular domains through focal

adhesion kinase, however (Table 7), and subsequently by MAPK or PI3K activation (Table 8) leading to multiple effects as outlined above.<sup>40,41</sup> Stewart *et al.*<sup>42</sup> provide an excellent review of changes in the extracellular matrix associated with prostate cancer progression. The data specifically related to castration is more limited. *In vitro*, fibronectin expression is increased over 60-fold in androgen-independent clones<sup>43</sup> and mucin expression also undergoes significant changes.<sup>44</sup> Current treatment strategies based on these observations are under development. The expression of mucin type 1 by neoplastic cells has inspired attempts at vaccine creation.<sup>45</sup> In addition, part of the putative activity of genistein in opposing neoplastic progression might be caused by alteration of signaling involving focal adhesion kinase.<sup>46</sup>

A factor that encompasses multiple effects is nuclear factor  $\kappa$ B (NF $\kappa$ B), a nuclear transcription factor (Table 9). Multiple pathways can phosphorylate inhibitor of  $\kappa$ B (I $\kappa$ B), the cytosolic binding protein for NF $\kappa$ B, causing the release of NF $\kappa$ B, which quickly translocates to the nucleus. Once in the nucleus, NF $\kappa$ B facilitates transcription of multiple genes associated with inflammation (e.g interleukins 1 and 6 and tumor necrosis factor- $\alpha$  [Table 5]), cell adhesion

**Table 9** Castration-induced changes in transcription factors possibly involved in androgen receptor-independent prostate cancer progression.

| Factor             | CaP related physiology  | Castration related changes | Potential clinical role  | Selected references |
|--------------------|---|----------------------------|--|---------------------|
| AR <sup>a</sup>    | Androgen binding induces dimerization, activating transcription factor activity for many genes <sup>a</sup> | Increased <sup>a</sup>     | Future: blocking downstream signals completely <sup>a</sup>                        | 3, 73               |
| c-Fos <sup>a</sup> | Combines with c-Jun to make AP-1 transcription factor <sup>a</sup>  | Variable <sup>a</sup>      | Prognostic factor <sup>a</sup>   | 90                  |
| c-Jun <sup>a</sup> | Dimerizes or combines with c-Fos to make AP-1 transcription factor <sup>a</sup>                             | Variable <sup>a</sup>      | Prognostic factor <sup>a</sup>   | 90                  |
| c-Myc              | Transcription factor; supports AI growth; inhibits apoptosis  | May be increased           | Phosphorodiamidate morpholino oligomers against c-Myc in early trials <sup>a</sup> | 82, 73              |
| NFκB               | Causes transcription of multiple factors involved in angiogenesis, metastasis, and antiapoptosis            | Increased                  | Proteasome inhibitor bortezomib opposes degradation of IκB <sup>a</sup>            | 71                  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: AI, androgen independent; AP-1, activating protein 1; AR, androgen receptor; CaP, prostate cancer; IκB, inhibitor of κB; NFκB, nuclear factor κB.

**Table 10** Castration-induced changes in miscellaneous factors possibly involved in androgen receptor-independent prostate cancer progression.

| Factor                              | CaP related physiology   | Castration related changes                                   | Potential clinical role                               | Selected references |
|-------------------------------------|--|--|---|---------------------|
| Arachidonic acid                    | Increased by EGF/neurotensin signaling                                 | Possible upregulation if NE cells switch to GF pathways      | COX-2 inhibitors show modest activity in early trials | 94                  |
| CD-117 (c-kit)                      | NR   | Induced in 1 metastatic sample                               | NR  | 64                  |
| COX-2                               | Enzyme produces prostaglandins from arachidonic acid                   | Increased  | COX-2 inhibitors show modest activity in early trials | 83, 94, 95          |
| Id-1                                | Upregulates EGFR; inhibits transcription factors                       | Increased  | Future target   | 96                  |
| Insulin receptor                    | Controls glucose balance   | Increased  | NR  | 61                  |
| RPTPα                               | Role in neuronal differentiation; activates Src                        | Increased  | Marker for NED and correlation with ERK1/2 activation | 72                  |
| SREBPs <sup>a</sup>                 | Regulate transcription of enzymes involved in lipogenesis <sup>a</sup> | Increased <sup>a</sup>                                       | NR  | 97                  |
| SREBP cleavage protein <sup>a</sup> | Cleaves SREBPs <sup>a</sup>  | Initial decrease; increased upon AI progression <sup>a</sup> | NR  | 97                  |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: AI, androgen independent; CaP, prostate cancer; COX, cyclo-oxygenase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated protein kinase; GF, growth factor; Id-1, inhibitor of DNA binding 1; NE, neuroendocrine; NED, neuroendocrine differentiation; NR, not reported; RPTPα, receptor-like tyrosine phosphatase α; SREBP, sterol regulatory element-binding proteins.

(e.g. vascular cell-adhesion molecule, inter-cellular cell-adhesion molecule and E-selectin), stress response (e.g. cyclooxygenase 2 [Table 10] and nitric oxide synthase), and factors inhibiting apoptosis (e.g. Bcl-2 family and inhibitor-of-apoptosis proteins [Table 6]).<sup>47</sup> These pathways are intimately involved in proliferation, angiogenesis, invasiveness, metastasis, and apoptosis inhibition.<sup>48</sup> Recently, bortezomib, an inhibitor of the proteasome responsible for degrading IκB, has shown efficacy and tolerability in prostate cancer and might be a promising treatment in combination with other agents.<sup>49,50</sup>

## CURRENT TREATMENTS FOR ANDROGEN-INDEPENDENT PROSTATE CANCER

Docetaxel was recently shown to modestly improve survival in androgen-independent prostate cancer.<sup>51,52</sup> Most of the biologic agents discussed earlier in this review are currently undergoing testing in combination with docetaxel at the time of androgen-independent disease progression. This strategy shows some promise, as many of the biologic agents show modest activity alone, seeming to function better as chemosensitizers or radiosensitizers.<sup>12</sup> By contrast, very few studies exist that examine

**Table 11** Castration-induced changes in prostate cancer markers in androgen receptor-independent prostate cancer progression.

| Factor                          | CaP related physiology                                | Castration related changes  | Potential clinical role                       | Selected references |
|---------------------------------|---|---|---|---------------------|
| CgA <sup>a</sup>                | Marker <sup>a</sup>                                   | Increase in plasma levels <sup>a</sup>                                  | Marker of NED <sup>a</sup>                    | 98                  |
| CgB                             | Marker  | 5–10 fold increase from NE cells  | Better marker than CgA in certain NED cancers | 72                  |
| Ki67 (MIB-1) <sup>a</sup>       | Nuclear antigen; marker of cell division <sup>a</sup> | Decreased or no change <sup>a</sup>                                     | Marker for cell division <sup>a</sup>         | 34, 64              |
| NSE <sup>a</sup>                | Marker <sup>a</sup>                                   | Variable <sup>a</sup>   | Prognostic marker <sup>a</sup>                | 72                  |
| PSA                             | Marker  | Decreased overall owing to cell death; decreased expression by NE cells | Prognostic marker                             | 72                  |
| PSP94 <sup>a</sup>              | Unknown <sup>a</sup>                                  | Persists during ADT <sup>a</sup>  | Marker of AI CaP <sup>a</sup>                 | 99                  |
| Tissue glutaminase <sup>a</sup> | Marker for apoptosis <sup>a</sup>                     | Initial increase followed by decrease <sup>a</sup>                      | Apoptosis marker <sup>a</sup>                 | 100                 |

<sup>a</sup>Studies in human subjects available for review. Abbreviations: ADT, androgen deprivation therapy; AI, androgen independent; CaP, prostate cancer; CgA, chromogranin A; CgB, chromogranin B; MIB-1, ubiquitin ligase MIB-1 (mindbomb homolog 1); NE, neuroendocrine; NED, neuroendocrine differentiation; NSE, neuron specific enolase; PSA, prostate-specific antigen; PSP94, prostate-specific protein-94.

the adjunctive use of these biologic agents at the time of castration. We believe that the changes occurring at the time of castration set in motion events leading to androgen-independent prostate cancer, thus indicating a role for adjunctive therapy at that time. The rationale for adjunctive treatment for prostate cancer at the time of castration is based on several lines of evidence.

First, microenvironmental changes in the cellular milieu are likely to have a major role in the initiation of clinical disease.<sup>53</sup> ADT induces further changes (listed in Tables 1–11), which, through poorly defined biochemical pathways, promote the emergence of androgen-independent prostate cancer. Future treatment strategies might target these microenvironmental cellular changes and attempt to reverse or ameliorate them through gene silencing (ASOs, small interfering RNA) or direct inactivation/activation of opposing factors (mAbs). Second, the apoptotic response to the act of castration is never complete, and efforts to enhance this response will decrease residual disease and possibly delay disease progression. Subsets of cells can have resistance to apoptosis through mutational changes in effectors (e.g. p53/p21) or through the chance occurrence of elevated precastration levels of antiapoptotic factors (e.g. Bcl family proteins or HSPs). Efforts, initiated at the time of castration, to increase the effectiveness of treatment and approximate a maximal apoptotic response should be attempted. Third, the smaller tumor

burden associated with earlier treatment of prostate cancer potentially allows a greater chance for effective intervention.<sup>28</sup> Finally, the biologic therapies discussed are attractive in that they generally have relatively low toxicity profiles, making them suitable adjunctive treatments for prostate cancer.

Of the many castration-induced changes listed in the tables presented in this review, mechanisms suggested for future study include those that are consistently seen *in vivo* and that have well-characterized and effective inhibitors available. Proteasome inhibition, targeted therapies such as PI3K inhibitors or Bcl-2 ASOs, and small molecule tyrosine kinase inhibitors all represent possible strategies for treating prostate cancer in the adjunctive setting. In addition, angiogenesis inhibitors and cellular adhesion modifications might decrease growth and metastasis as well as having direct intracellular effects on a variety of pathways.

## CONCLUSION

Increasing the effectiveness of ADT and/or delaying the onset of androgen-independent prostate cancer represents a major treatment strategy for this disease. The biochemical changes demonstrated by prostate cancer cells undergoing castration suggest therapeutic methods for adjunctive treatment. Specific therapies currently exist that target many of these pathways. These should ultimately be tested in the adjunctive setting.

## KEY POINTS

- Although not completely understood, the progression of prostate cancer to an androgen-independent state probably involves multiple biochemical pathways
- Therapeutic androgen ablation is likely to be an initial factor driving this biochemical cascade of events
- Novel biologic agents are now available enabling modification of some of the pathways involved in the development of androgen-independent prostate cancer
- Current clinical data shows some efficacy for biologic agents when used with chemotherapy in the setting of androgen-independent disease
- Future trials should also test biologic agents at the time of androgen ablation to attempt to maximize the initial apoptotic response and delay the onset of androgen independence

## References

- 1 Jemal A *et al.* (2006) Cancer statistics, 2006. *CA Cancer J Clin* 56: 106–130
- 2 Smaletz O *et al.* (2002) Nomogram for overall survival of patients with progressive metastatic prostate cancer after castration. *J Clin Oncol* 20: 3972–3982
- 3 Sharifi N and Farrar WL (2006) Androgen receptor as a therapeutic target for androgen independent prostate cancer. *Am J Ther* 13: 166–170
- 4 Feldman BJ and Feldman D (2001) The development of androgen-independent prostate cancer. *Nat Rev Cancer* 1: 34–45
- 5 So A *et al.* (2005) Mechanisms of the development of androgen independence in prostate cancer. *World J Urol* 23: 1–9
- 6 Wu RC *et al.* (2005) Transcriptional regulation by steroid receptor coactivator phosphorylation. *Endocr Rev* 26: 393–399
- 7 Culig Z *et al.* (2004) Expression and function of androgen receptor coactivators in prostate cancer. *J Steroid Biochem Mol Biol* 92: 265–271
- 8 Gioeli D *et al.* (1999) Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res* 59: 279–284
- 9 Lorenzo GD *et al.* (2003) Involvement of growth factor receptors of the epidermal growth factor receptor family in prostate cancer development and progression to androgen independence. *Clin Prostate Cancer* 2: 50–57
- 10 Lara PN Jr *et al.* (2004) Trastuzumab plus docetaxel in HER-2/neu-positive prostate carcinoma: final results from the California Cancer Consortium Screening and Phase II Trial. *Cancer* 100: 2125–2131
- 11 Ziada A *et al.* (2004) The use of trastuzumab in the treatment of hormone refractory prostate cancer; phase II trial. *Prostate* 60: 332–337
- 12 van der Poel HG (2004) Smart drugs in prostate cancer. *Eur Urol* 45: 1–17
- 13 Smith MR and Nelson JB (2005) Future therapies in hormone-refractory prostate cancer. *Urology* 65: 9–16; discussion 17
- 14 Canil CM *et al.* (2005) Randomized phase II study of two doses of gefitinib in hormone-refractory prostate cancer: a trial of the National Cancer Institute of Canada-Clinical Trials Group. *J Clin Oncol* 23: 455–460
- 15 Krueckl SL *et al.* (2004) Increased insulin-like growth factor I receptor expression and signaling are components of androgen-independent progression in a lineage-derived prostate cancer progression model. *Cancer Res* 64: 8620–8629
- 16 Miyake H *et al.* (2000) Castration-induced up-regulation of insulin-like growth factor binding protein-5 potentiates insulin-like growth factor-I activity and accelerates progression to androgen independence in prostate cancer models. *Cancer Res* 60: 3058–3064
- 17 Kiyama S *et al.* (2003) Castration-induced increases in insulin-like growth factor-binding protein 2 promotes proliferation of androgen-independent human prostate LNCaP tumors. *Cancer Res* 63: 3575–3584
- 18 Miyata Y *et al.* (2004) Expression of insulin-like growth factor binding protein-3 before and after neoadjuvant hormonal therapy in human prostate cancer tissues: correlation with histopathologic effects and biochemical recurrence. *Urology* 63: 1184–1190
- 19 Papandreou CN *et al.* (1998) Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nat Med* 4: 50–57
- 20 Lee LF *et al.* (2001) Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. *Mol Cell Biol* 21: 8385–8397
- 21 Amorino GP and Parsons SJ (2004) Neuroendocrine cells in prostate cancer. *Crit Rev Eukaryot Gene Expr* 14: 287–300
- 22 Song L *et al.* (2006) Dasatinib (BMS-354825) selectively induces apoptosis in lung cancer cells dependent on epidermal growth factor receptor signaling for survival. *Cancer Res* 66: 5542–5548
- 23 Yang JC *et al.* (2005) Src kinase inhibition of neuropeptide-induced androgen-independent prostate cancer. *Proc Amer Assoc Cancer Res* 46: 3180
- 24 Nam S *et al.* (2005) Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res* 65: 9185–9189
- 25 McDonnell TJ *et al.* (1992) Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 52: 6940–6944
- 26 Colombel M *et al.* (1993) Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. *Am J Pathol* 143: 390–400
- 27 Shi Y *et al.* (2006) Role of coordinated molecular alterations in the development of androgen-independent prostate cancer: an *in vitro* model that corroborates clinical observations. *BJU Int* 97: 170–178
- 28 Miyake H *et al.* (2001) Novel therapeutic strategy for advanced prostate cancer using antisense oligodeoxynucleotides targeting anti-apoptotic genes upregulated after androgen withdrawal to delay androgen-independent progression and enhance chemosensitivity. *Int J Urol* 8: 337–349
- 29 Tolcher AW *et al.* (2005) A phase II, pharmacokinetic, and biological correlative study of oblimersen sodium and docetaxel in patients with hormone-refractory prostate cancer. *Clin Cancer Res* 11: 3854–3861
- 30 Gleave M *et al.* (2005) Beyond simple castration: targeting the molecular basis of treatment resistance in advanced prostate cancer. *Cancer Chemother Pharmacol* 56 (Suppl 1): 47–57
- 31 July LV *et al.* (2002) Clusterin expression is significantly enhanced in prostate cancer cells following androgen withdrawal therapy. *Prostate* 50: 179–188
- 32 Miyake H *et al.* (2005) Antisense oligodeoxynucleotide therapy targeting clusterin gene for prostate cancer: Vancouver experience from discovery to clinic. *Int J Urol* 12: 785–794

- 33 Chi KN *et al.* (2005) A phase I pharmacokinetic and pharmacodynamic study of OGX-011, a 2'-methoxyethyl antisense oligonucleotide to clusterin, in patients with localized prostate cancer. *J Natl Cancer Inst* **97**: 1287–1296
- 34 Baretton GB *et al.* (1999) Proliferation- and apoptosis-associated factors in advanced prostatic carcinomas before and after androgen deprivation therapy: prognostic significance of p21/WAF1/CIP1 expression. *Br J Cancer* **80**: 546–555
- 35 Wikstrom P *et al.* (1999) Early castration-induced upregulation of transforming growth factor beta1 and its receptors is associated with tumor cell apoptosis and a major decline in serum prostate-specific antigen in prostate cancer patients. *Prostate* **38**: 268–277
- 36 Chevalier S *et al.* (2002) Vascular endothelial growth factor and signaling in the prostate: more than angiogenesis. *Mol Cell Endocrinol* **189**: 169–179
- 37 George DJ *et al.* (2001) Prognostic significance of plasma vascular endothelial growth factor levels in patients with hormone-refractory prostate cancer treated on Cancer and Leukemia Group B 9480. *Clin Cancer Res* **7**: 1932–1936
- 38 Lara PN Jr *et al.* (2004) Angiogenesis-targeted therapies in prostate cancer. *Clin Prostate Cancer* **3**: 165–173
- 39 Picus J *et al.* (2003) The use of bevacizumab (B) with docetaxel (D) and estramustine (E) in hormone refractory prostate cancer (HRPC): Initial results of CALGB 90006. *Proc Am Soc Clin Oncol* **22**: 393
- 40 Schlaepfer DD and Hunter T (1998) Integrin signalling and tyrosine phosphorylation: just the FAKs? *Trends Cell Biol* **8**: 151–157
- 41 Danen EH and Yamada KM (2001) Fibronectin, Integrins, and growth control. *J Cell Physiol* **189**: 1–13
- 42 Stewart DA *et al.* (2004) Changes in extracellular matrix (ECM) and ECM-associated proteins in the metastatic progression of prostate cancer. *Reprod Biol Endocrinol* **2**: 2
- 43 Stubbs AP *et al.* (1999) Differentially expressed genes in hormone refractory prostate cancer: association with chromosomal regions involved with genetic aberrations. *Am J Pathol* **154**: 1335–1343
- 44 Legrier ME *et al.* (2004) Mucinous differentiation features associated with hormonal escape in a human prostate cancer xenograft. *Br J Cancer* **90**: 720–727
- 45 North SA *et al.* (2006) A pilot study of the liposomal MUC1 vaccine BLP25 in prostate specific antigen failures after radical prostatectomy. *J Urol* **176**: 91–95
- 46 Kousidou O *et al.* (2006) Effects of the natural isoflavonoid genistein on growth, signaling pathways and gene expression of matrix macromolecules by breast cancer cells. *Mini Rev Med Chem* **6**: 331–337
- 47 Papandreou CN and Logothetis CJ (2004) Bortezomib as a potential treatment for prostate cancer. *Cancer Res* **64**: 5036–5043
- 48 Orlowski RZ and Baldwin AS Jr (2002) NF-kappaB as a therapeutic target in cancer. *Trends Mol Med* **8**: 385–389
- 49 Price N and Dreicer R (2004) Phase I/II trial of bortezomib plus docetaxel in patients with advanced androgen-independent prostate cancer. *Clin Prostate Cancer* **3**: 141–143
- 50 Papandreou CN *et al.* (2004) Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer. *J Clin Oncol* **22**: 2108–2121
- 51 Petrylak DP *et al.* (2004) Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* **351**: 1513–1520
- 52 Tannock IF *et al.* (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* **351**: 1502–1512
- 53 Chung LW *et al.* (2005) Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol* **173**: 10–20
- 54 Gregory CW *et al.* (1998) Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res* **58**: 5718–5724
- 55 Hong H *et al.* (2004) Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status. *Cancer* **101**: 83–89
- 56 Debes JD *et al.* (2002) p300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6. *Cancer Res* **62**: 5632–5636
- 57 Halkidou K *et al.* (2003) Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. *Oncogene* **22**: 2466–2477
- 58 Culig Z *et al.* (1994) Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* **54**: 5474–5478
- 59 Dorkin TJ *et al.* (1999) aFGF immunoreactivity in prostate cancer and its co-localization with bFGF and FGF8. *J Pathol* **189**: 564–569
- 60 Sirotinak FM *et al.* (2004) Microarray analysis of prostate cancer progression to reduced androgen dependence: studies in unique models contrasts early and late molecular events. *Mol Carcinog* **41**: 150–163
- 61 Bubendorf L *et al.* (1999) Hormone therapy failure in human prostate cancer: analysis by complementary DNA and tissue microarrays. *J Natl Cancer Inst* **91**: 1758–1764
- 62 Stewart RJ *et al.* (2001) Vascular endothelial growth factor expression and tumor angiogenesis are regulated by androgens in hormone responsive human prostate carcinoma: evidence for androgen dependent destabilization of vascular endothelial growth factor transcripts. *J Urol* **165**: 688–693
- 63 Aslan G *et al.* (2005) Vascular endothelial growth factor expression in untreated and androgen-deprived patients with prostate cancer. *Pathol Res Pract* **201**: 593–598
- 64 Zellweger T *et al.* (2005) Expression patterns of potential therapeutic targets in prostate cancer. *Int J Cancer* **113**: 619–628
- 65 Naimi B *et al.* (2002) Down-regulation of (IIb) and (IIc) isoforms of fibroblast growth factor receptor 2 (FGFR2) is associated with malignant progression in human prostate. *Prostate* **52**: 245–252
- 66 Dizzei N *et al.* (2005) Expression of serotonin receptors 2B and 4 in human prostate cancer tissue and effects of their antagonists on prostate cancer cell lines. *Eur Urol* **47**: 895–900
- 67 Jongsma J *et al.* (2000) Androgen deprivation of the PC-310 [correction of prohormone convertase-310] human prostate cancer model system induces neuroendocrine differentiation. *Cancer Res* **60**: 741–748
- 68 Salido M *et al.* (2002) Neuropeptides bombesin and calcitonin inhibit apoptosis-related elemental changes in prostate carcinoma cell lines. *Cancer* **94**: 368–377
- 69 Takahashi W *et al.* (2003) Regulatory effect of castration on endothelins, their receptors and endothelin-converting enzyme in rat seminal vesicle. *BJU Int* **92**: 803–809
- 70 Yashi M *et al.* (2003) Elevated serum progastrin-releasing peptide (31-98) level is a predictor of short response duration after hormonal therapy in metastatic prostate cancer. *Prostate* **56**: 305–312
- 71 Levine L *et al.* (2003) Bombesin stimulates nuclear factor kappa B activation and expression of

**Acknowledgments**

Research supported in part by NIH grant KO8 DK60748-01 and Department of Defense grant PC040161.

**Competing interests**

The authors declared they have no competing interests.

- proangiogenic factors in prostate cancer cells. *Cancer Res* 63: 3495–3502
- 72 Yuan TC *et al.* (2006) Androgen deprivation induces human prostate epithelial neuroendocrine differentiation of androgen-sensitive LNCaP cells. *Endocr Relat Cancer* 13: 151–167
  - 73 Vashchenko N and Abrahamsson PA (2005) Neuroendocrine differentiation in prostate cancer: implications for new treatment modalities. *Eur Urol* 47: 147–155
  - 74 Gutierrez-Canas I *et al.* (2005) Vasoactive intestinal peptide induces neuroendocrine differentiation in the LNCaP prostate cancer cell line through PKA, ERK, and PI3K. *Prostate* 63: 44–55
  - 75 Sugihara A *et al.* (1998) Expression of cytokines enhancing the osteoclast activity, and parathyroid hormone-related protein in prostatic cancers before and after endocrine therapy: an immunohistochemical study. *Oncol Rep* 5: 1389–1394
  - 76 Wise GJ *et al.* (2000) Cytokine variations in patients with hormone treated prostate cancer. *J Urol* 164: 722–725
  - 77 Lee LF *et al.* (2004) Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene* 23: 2197–2205
  - 78 Huerta-Yepez S *et al.* (2006) Involvement of the TNF- $\alpha$  autocrine-paracrine loop, via NF- $\kappa$ B and YY1, in the regulation of tumor cell resistance to Fas-induced apoptosis. *Clin Immunol* 120: 297–309
  - 79 Watson RW and Fitzpatrick JM (2005) Targeting apoptosis in prostate cancer: focus on caspases and inhibitors of apoptosis proteins. *BJU Int* 96 (Suppl 2): 30–34
  - 80 Koivisto PA and Rantala I (1999) Amplification of the androgen receptor gene is associated with P53 mutation in hormone-refractory recurrent prostate cancer. *J Pathol* 187: 237–241
  - 81 Pflug BR *et al.* (1999) Caveolin expression is decreased following androgen deprivation in human prostate cancer cell lines. *Prostate* 40: 269–273
  - 82 Persad S and Dedhar S (2003) The role of integrin-linked kinase (ILK) in cancer progression. *Cancer Metastasis Rev* 22: 375–384
  - 83 Miyamoto H *et al.* (2005) Inhibition of the Akt, cyclooxygenase-2, and matrix metalloproteinase-9 pathways in combination with androgen deprivation therapy: potential therapeutic approaches for prostate cancer. *Mol Carcinog* 44: 1–10
  - 84 Kiviniemi J *et al.* (2004) Altered expression of syndecan-1 in prostate cancer. *Apmis* 112: 89–97
  - 85 Lokeshwar BL (1999) MMP inhibition in prostate cancer. *Ann N Y Acad Sci* 878: 271–289
  - 86 Bratland A *et al.* (2003) The metalloproteinase inhibitor TIMP-2 is down-regulated by androgens in LNCaP prostate carcinoma cells. *Clin Exp Metastasis* 20: 541–547
  - 87 Nishiyama T *et al.* (2004) The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. *Clin Cancer Res* 10: 7121–7126
  - 88 Lei Q *et al.* (2006) NIKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss. *Cancer Cell* 9: 367–378
  - 89 Majumder PK and Sellers WR (2005) Akt-regulated pathways in prostate cancer. *Oncogene* 24: 7465–7474
  - 90 Edwards J *et al.* (2004) The role of c-Jun and c-Fos expression in androgen-independent prostate cancer. *J Pathol* 204: 153–158
  - 91 Aprikian AG *et al.* (1996) Bombesin specifically induces intracellular calcium mobilization via gastrin-releasing peptide receptors in human prostate cancer cells. *J Mol Endocrinol* 16: 297–306
  - 92 Sumitomo M *et al.* (2001) Neutral endopeptidase inhibits neuropeptide-mediated transactivation of the insulin-like growth factor receptor-Akt cell survival pathway. *Cancer Res* 61: 3294–3298
  - 93 Devi GR *et al.* (2005) *In vivo* bioavailability and pharmacokinetics of a c-MYC antisense phosphorodiamidate morpholino oligomer, AVI-4126, in solid tumors. *Clin Cancer Res* 11: 3930–3938
  - 94 Smith MR *et al.* (2006) Celecoxib versus placebo for men with prostate cancer and a rising serum prostate-specific antigen after radical prostatectomy and/or radiation therapy. *J Clin Oncol* 24: 2723–2728
  - 95 Pruthi RS *et al.* (2003) Cyclooxygenase-2 as a potential target in the prevention and treatment of genitourinary tumors: a review. *J Urol* 169: 2352–2359
  - 96 Ling MT *et al.* (2004) Id-1 expression induces androgen-independent prostate cancer cell growth through activation of epidermal growth factor receptor (EGF-R). *Carcinogenesis* 25: 517–525
  - 97 Ettinger SL *et al.* (2004) Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence. *Cancer Res* 64: 2212–2221
  - 98 Sasaki T *et al.* (2005) Changes in chromogranin a serum levels during endocrine therapy in metastatic prostate cancer patients. *Eur Urol* 48: 224–229; discussion 229–230
  - 99 Imasato Y *et al.* (2000) PSP94 expression after androgen deprivation therapy: a comparative study with prostate specific antigen in benign prostate and prostate cancer. *J Urol* 164: 1819–1824
  - 100 Rittmaster RS *et al.* (1999) The utility of tissue transglutaminase as a marker of apoptosis during treatment and progression of prostate cancer. *J Urol* 162: 2165–2169

## REVIEW

# Inhibition of Akt pathways in the treatment of prostate cancer

EC Nelson<sup>1</sup>, CP Evans<sup>1,2</sup>, PC Mack<sup>2</sup>, RW Devere-White<sup>1,2</sup> and PN Lara Jr<sup>2</sup>

<sup>1</sup>Department of Urology, University of California at Davis, Sacramento, CA, USA and <sup>2</sup>Cancer Center, University of California at Davis, Sacramento, CA, USA

**Akt is a serine/threonine kinase mediating multiple intracellular pathways involved in prostate cancer (CaP) biology. Increased understanding of the molecular mechanisms of Akt activation and signaling have led to the development of an increasing number of Akt inhibitors. These biologic agents demonstrate activity against a wide range of cancers in preclinical studies. Clinical studies of Akt inhibition in CaP are in progress, including agents such as celecoxib, perifosine and genistein. How best to integrate Akt inhibitors with standard CaP therapy or select patients most likely to benefit is the subject of ongoing research.**

*Prostate Cancer and Prostatic Diseases* (2007) 10, 331–339; doi:10.1038/sj.pcan.4500974; published online 1 May 2007

**Keywords:** akt; celecoxib; genistein; perifosine; protein kinase B

## Introduction

Akt, or protein kinase B, is a serine/threonine kinase that plays an important role in intracellular signaling cascades. A variety of neoplasms show perturbations in the biochemical pathways affected by Akt. Prostate cancer (CaP) specifically shows biochemical abnormalities related to Akt that may be of importance in sustaining tumor growth by preventing apoptosis and promoting proliferation and angiogenesis.

CaP is the most common noncutaneous malignancy in American males and is predicted to be the third leading cause of cancer deaths for 2006.<sup>1</sup> While local therapy for CaP is relatively effective, androgen deprivation therapy remains the mainstay of treatment for disseminated disease and is principally palliative in nature. Introduced in the 1940s,<sup>2</sup> androgen deprivation removes androgen stimulation, initially inducing apoptosis in CaP. However, the disease eventually progresses to an androgen independent (AI) state with an associated life expectancy of only 15–20 months. Androgen deprivation, while extending length and quality of life for many patients, also induces tumor-specific biochemical changes of many intracellular factors including Akt. These changes may promote progression to an AI state.<sup>3</sup>

Novel treatments for AI CaP are needed. Increasing understanding of the many biochemical changes associated with neoplastic progression and androgen independence has led to the identification of novel targets for therapeutic intervention. In this review, we discuss

pathways relating directly to Akt, focusing on those showing the greatest relevance to current and possible future therapeutic strategies.

## Pathways affected by Akt

### *Akt form and function*

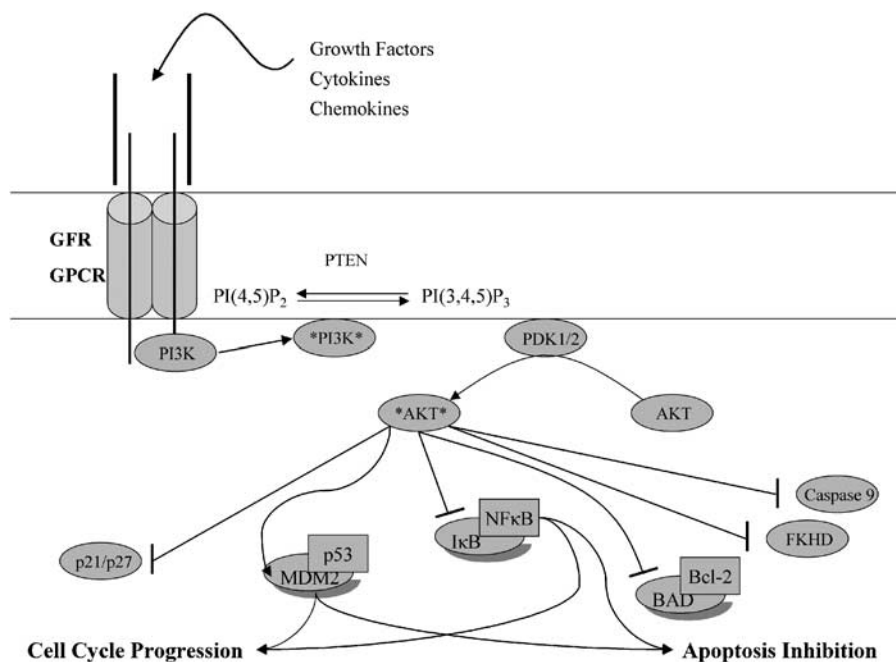
Akt was originally identified as an oncogene within the AKT8 retrovirus. This retrovirus was isolated from the AKR strain of mice that have a high incidence of leukemia and lymphoma.<sup>4</sup> Subsequent genetic analysis demonstrated that Akt is an important intracellular signaling moiety highly conserved across species. A member of the AGC kinase family, it is very similar to protein kinase A and protein kinase C. When first discovered, it was therefore named 'protein kinase B' and is sometimes called RAC (related to A and C).

In humans, Akt is a family of three homologous members out of which Akt1 and Akt2 are more widely distributed than Akt3.<sup>5</sup> Akt has three domains with specific functions. The N-terminal domain is a pleckstrin homology (PH) domain, which can bind phosphoinositides (PI) in the cellular membrane. The C-terminal domain is a regulatory domain and the central portion of the protein is the catalytic domain.<sup>6</sup> Complete activation of the catalytic activity of Akt requires phosphorylation of a threonine residue at 308 and a serine residue at 473. It is possible that Akt shows partial activation with phosphorylation at the threonine 308 position.<sup>7</sup>

### *Akt activation*

Akt activation occurs in response to multiple extracellular signals acting through tyrosine kinase and G-protein coupled receptors (see Figure 1). These receptor types activate phosphoinositol-3-kinase (PI3K) class IA

Correspondence: Dr PN Lara Jr, Division of Hematology/Oncology, Department of Internal Medicine, School of Medicine, University of California, Davis, 4501 X Street, Sacramento, CA 95817, USA.  
E-mail: primo.lara@ucdmc.ucdavis.edu  
Received 17 January 2007; revised 29 March 2007; accepted 30 March 2007; published online 1 May 2007



**Figure 1** Simplified diagram of Akt activation and selected downstream effector pathways. Multiple extracellular signaling factors activate intracellular receptor domains. Activated PI3K promotes the 3' phosphorylation of PI(4,5)P<sub>2</sub>. The resulting PI(3,4,5)P<sub>3</sub> recruits Akt and PDK1 to the cell membrane through interactions with their PH domains. Akt is activated by phosphorylation resulting in multiple downstream effects. Abbreviations: GFR, growth factor receptor; GPCR, G-protein coupled receptor; NFκB, nuclear factor kappa B; PDK, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide-3-kinase; PI(3,4,5)P<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; PI(4,5)P<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

and IB, respectively. In turn, PI3K phosphorylates the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PI(4,5)P<sub>2</sub>), which then acts as the second-messenger phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) for a variety of pathways. This event promotes recruitment of Akt from the cytoplasm to the cellular membrane where phosphoinositide-dependent kinase-1 (PDK1) phosphorylates the threonine 308 position of Akt. Phosphorylation of the serine 473 position also occurs, although the kinase responsible has not been definitively identified. This step appears to be tightly regulated and may require multiple intracellular messengers including integrin-linked kinase, PDK2 and others.<sup>7</sup>

#### Upstream inhibitors

An intricate web of inhibitory factors opposing the actions of PI3K and subsequent second messengers control regulation of Akt activation. Most notably, 'phosphatase and tensin homolog deleted on chromosome 10' (PTEN) directly opposes PI3K by removing the 3'-phosphate from PI(3,4,5)P<sub>3</sub>. A recently discovered class of phosphatases called SHIP phosphatases also may play a role in controlling levels of PI(3,4,5)P<sub>3</sub>, and thus subsequent Akt activation. However it appears that as SHIP removes the 5-phosphate from PI(3,4,5)P<sub>3</sub>, the resultant PI(3,4)P<sub>2</sub> may still recruit Akt to the plasma membrane. Therefore, PTEN is probably the more important inhibitor.<sup>5</sup> Other recently discovered phosphatases include C-terminal modulator protein and PH domain leucine-rich repeat protein phosphatase, both of which may be significant in Akt regulation.<sup>8</sup>

Direct deactivation of Akt is also possible. Protein phosphatases (PP1 and PP2a) both govern the regulatory activity of many intracellular messengers including Akt through dephosphorylation. The complex activity of these regulatory proteins is controlled through cell wall constituents including palmitate, integrin and caveolin.<sup>5</sup> Heat shock protein 90 may oppose the actions of these phosphatases, thereby promoting Akt activity.<sup>9</sup>

#### Akt downstream effects

Following activation, Akt moves from the cellular membrane to the cytoplasm where it exercises broad control over a variety of intracellular pathways generally supporting survival, proliferation, and other activities necessary for neoplastic disease progression.

**Apoptosis and the cell cycle.** Cancer cells escape normal biochemical systems regulating the balance between apoptosis and survival. Akt generally acts to promote survival through inhibition of proapoptotic factors and activation of anti-apoptotic factors. For example, the Bcl-2 family of proteins consists of both proapoptotic and anti-apoptotic factors, the balance of which is critical for maintaining cellular homeostasis. Through phosphorylation, Akt inhibits the activity of proapoptotic members such as BAD, BAX and BID while activating anti-apoptotic members such as Bcl-xL.<sup>10-12</sup>

Another family of apoptotic regulators is the forkhead family. In general, Akt phosphorylates various members of this family causing translocation from the nucleus to

the cytoplasm, thus inhibiting the transcription of proapoptotic genes.<sup>7</sup>

Other biochemical pathways affected include apoptosis-signal-regulating kinase cyclic AMP response element binding protein, and the oncoprotein MDM2. Effects through these include inhibition of jun N-terminal kinase and p53. In addition, Akt may block apoptosis after it has been initiated. For example, Akt can prevent the activation of caspase-9 despite mitochondrial cytochrome *c* release.<sup>5</sup>

Cancer cells also escape normal cellular controls over the cell cycle, generally resulting in increased, deregulated proliferation. Akt activation may promote this process through multiple pathways. Three central regulators of the cell cycle affected by Akt are cyclin D, p21 and p27. Cyclin D is necessary for cyclin-dependent kinase (CDK) activity regulating entry into the cell cycle. p21 and p27 are CDK inhibitors, which oppose cell-cycle progression. Akt phosphorylates and inactivates p21 and p27 thereby eliminating a critical negative regulator of CDK activity and promoting progression through the cell cycle.<sup>5</sup>

## Other/multiple pathways

Other necessary cellular characteristics for neoplastic growth include angiogenesis, invasion and metastasis. Biochemical pathways affected by Akt may accomplish these effects with significant changes noted when Akt signaling intensity changes. In addition, three pathways discussed here have multiple or complex effects.

Akt activation may lead to increased angiogenesis through phosphorylation of endothelial nitric oxide synthase and subsequent production of nitric oxide.<sup>13,14</sup> In addition, Akt is a key activator of the mammalian target of rapamycin (mTOR) which, through stabilization of the hypoxia inducible factor, induces expression of pro-angiogenic genes such as vascular endothelial growth factor. mTOR also promotes cell survival and proliferation through other pathways. Examples of these include activation of p70 ribosomal S6 kinase and inhibition of 4E-BP1, thus promoting ribosomal translation in general, and increased expression of cyclin D, which promotes cellular cycling and proliferation.<sup>5</sup>

One cellular pathway affected by Akt with multiple effects is the nuclear factor kappa-B (NFκB) pathway. Akt causes the NFκB binding protein, IκB, to release NFκB, which then translocates to the nucleus where it transcribes multiple genes involved with proliferation, inflammation, cell adhesion, stress response and anti-apoptosis.<sup>15</sup> NFκB also increases expression of matrix metalloproteinases, which are frequently elevated in CaP specimens and may play a role in promoting invasion and metastasis.

p53 is a tumor suppressor showing aberrant regulation or mutation in many neoplasms. Akt may play a role in regulating its activity through activation of its binding protein, MDM2. Phosphorylation of MDM2 causes translocation to the nucleus where it inactivates p53 resulting in cell-cycle progression and inhibition of apoptosis.<sup>16</sup>

Of special interest in CaP is the observation that Akt can directly phosphorylate the androgen receptor on

serine residues at positions 210 and 790. The results of this are controversial with some authors reporting activation while others report suppression of androgen receptor signaling.<sup>17,18</sup> The reason for this may relate to different cell passage numbers, which is an interesting concept in the context of a rapidly dividing neoplasm.<sup>19</sup>

### *Alterations of Akt activity in CaP*

Increased Akt activity in CaP may be caused by genetic overexpression of Akt or altered expression of its upstream-positive and upstream-negative regulators. In CaP, several such mechanisms are probably active.

Akt overexpression has been demonstrated in CaP.<sup>20</sup> However, the most consistent finding in this disease is the silencing of PTEN and subsequent increase in Akt signaling.<sup>8</sup> PTEN may be lost by deletion, mutation or epigenetic mechanisms.<sup>5</sup> Up to half of the patients CaP tissue specimens show inactivation of PTEN with increasing incidence of this finding in metastatic deposits and AI disease, emphasizing its possible importance in tumor progression.<sup>21–23</sup> Other genetic overexpression or underexpression of factors upstream of Akt have also been demonstrated in CaP. Various growth factor receptors including the fibroblast growth factor, epidermal growth factor, and insulin-like growth factor are overexpressed in some CaP leading to increased Akt signaling.<sup>5</sup> In addition, PI3K may be overexpressed in CaP,<sup>5</sup> and may be important in the progression to AI disease.<sup>24</sup>

Regardless of the molecular mechanisms responsible, the excessive activation of Akt is a poor prognostic factor in CaP. In one report, phosphorylation of Akt was superior to measurements of cellular proliferation and even Gleason grade for predicting biochemical recurrence following radical prostatectomy.<sup>25</sup>

Treatments for CaP may also upregulate Akt pathways through activation of cellular stress responses. In CaP specifically, androgen withdrawal may lead to biochemical changes ultimately supporting the emergence of AI disease. Although Akt itself may not be directly involved in causing this transition, some of these pathways signal through Akt.<sup>3,26</sup> For example, emergence of a neuroendocrine phenotype in CaP may be important in disease progression. Such neuroendocrine cells may convert CaP cells to a dependence on survival signals through G-protein coupled receptors and growth factor receptors upstream of Akt, bypassing the usual androgen receptor signaling.<sup>26</sup>

These data cumulatively provide a rationale for Akt inhibition as a therapeutic paradigm in CaP.

### *Feedback mechanisms*

In light of the pathways and effects of Akt activation discussed above, it seems that Akt inhibition would naturally lead to positive therapeutic benefits in CaP and other neoplasms. Unfortunately, feedback mechanisms inherent in this complex biologic system may cause paradoxical responses to inhibition at various levels of Akt pathways. Two recently discovered feedback mechanisms demonstrate that inhibition of mTOR may, in fact, increase signaling through the Akt pathway.<sup>27</sup> Although not yet demonstrated *in vivo*, the existence of such complexity demonstrates a clear need for future

clinical trials to carefully measure the biologic effects of new therapies at the molecular level. Another factor complicating treatment using Akt inhibitors is one of the most important pathway for normal cellular physiology. It is not yet clear that developing treatment will demonstrate significant efficacy with acceptable levels of toxicity. These facts may lead to the use of Akt inhibition as adjunctive treatment rather than monotherapy.

## Akt inhibition as a therapeutic strategy

Data demonstrating the importance of increased Akt signaling pathways in supporting prostatic growth and the neoplastic progression of CaP have stimulated efforts to modulate these pathways through direct and indirect Akt inhibition. In view of the increased activation of Akt during some treatments for CaP, inhibition of Akt may be an important strategy for adjunctive therapy. Multiple inhibitors have been developed using a variety of mechanisms. Inhibition of PDK1 prevents activation of Akt and several effective agents are available (see Table 1). Direct inhibition of Akt may target any of the three domains discussed above using competitive, allosteric, pseudosubstrate or other mechanisms (see Table 2). Preclinical data on many Akt inhibitors are available and are reviewed in detail elsewhere.<sup>28–31</sup> Additional data regarding the Akt inhibitory properties of several nutraceuticals is emerging and may prove important in the future. Examples include quercetin,<sup>32</sup> diallyl trisulfide,<sup>33</sup> curcumin<sup>34</sup> and silymarin.<sup>35</sup> This review will be limited to agents for which clinical data are now available.

## Selective inhibitors of Akt

### *Celecoxib*

Celecoxib is a potent inhibitor of the inducible enzyme cyclooxygenase-2 (COX-2). By selective inhibition of COX-2 and avoidance of interference with the constitutively active COX-1, it was thought that celecoxib and other selective COX-2 inhibitors might be an effective treatment for inflammatory conditions while avoiding the gastric complications of long-term COX-1 inhibition. Although subsequent testing revealed an association with adverse cardiac outcomes leading to cessation of some ongoing trials, the drug remains on the market.

Celecoxib is currently of interest as preclinical experiments demonstrate significant proapoptotic effects in CaP cell lines. The biochemical activity of the drug is due to prevention of Akt phosphorylation by inhibiting the

action of PDK1<sup>27,36</sup> and this activity is independent of the COX-2 inhibitory effects.<sup>37</sup> In addition, COX-2 inhibitors may have other cellular functions potentiating the apoptotic response.<sup>38</sup> A therapeutic window for celecoxib might exist as the COX-2 enzyme is preferentially expressed in cancer tissue in response to tumor promoters, cytokines and growth factors.<sup>36</sup> However, some experiments show expression of induced COX-2 in CaP to be low if present, especially compared to other epithelial malignancies.<sup>39</sup> Although controversy exists on this point, COX-1 and -2 expression might be higher in the prostate in general regardless of disease processes.<sup>40</sup>

Outcomes data up to 20 years ago indicated a cancer chemopreventive effect for anti-inflammatory medications.<sup>41</sup> Large epidemiologic studies have examined this effect in celecoxib in a variety of cancers.<sup>42</sup> Specifically, the rationale for CaP chemoprevention using COX-2 inhibition was reviewed by Basler and Piazza.<sup>43</sup> Although no current clinical chemoprevention data are available, the use of celecoxib as adjunctive therapy merits attention.

A phase II study by Pruthi *et al.*<sup>44</sup> of celecoxib monotherapy to modify prostate-specific antigen (PSA) doubling time (PSADT) in patients with biochemical relapse following definitive therapy has been reported. Forty patients were enrolled, nineteen of whom had a PSADT of less than 6 months. Following treatment, 36 of 40 patients showed a declining PSADT, and 11 of 40 had their PSA decline with an additional 8 of 40 showing stable PSA values. A following randomized, placebo-controlled trial of this effect was terminated early based on the question of celecoxib safety. An *ad hoc* analysis of existing data on 78 randomized patients revealed a greater than 200% increase in PSADT in 40% of patients receiving celecoxib compared to 20% receiving placebo ( $P = 0.08$ ).<sup>45</sup>

Recent phase II studies demonstrate the use of celecoxib in combination with docetaxel and zoledronate.<sup>46,47</sup> Both the studies demonstrated biochemical and objective tumor responses. Another randomized, blinded trial of celecoxib as neoadjuvant therapy before prostatectomy showed activity in the disease. Significant effects on cellular signaling, oxidative stress and cell-cycle regulation were apparent upon blinded in comparison of the pathology specimens.<sup>48</sup>

In summary, preclinical data suggest a role for celecoxib in the treatment of CaP. Its apoptotic effects are mediated through inhibition of Akt phosphorylation by antagonism of PDK1. A therapeutic window may allow efficacy and development of derivatives will further refine the specificity of this medication.<sup>27</sup>

**Table 1** Select PDK-1 inhibitors

| Name               | IC <sub>50</sub> /L | Comment  | Selected citations |
|--------------------|---------------------|--|--------------------|
| Celecoxib          | 3.5–48 $\mu$ M      | COX-2 inhibitor  | 36,37,72,73        |
| DMC                | 38 $\mu$ M          | Celecoxib analog w/o COX-2 activity                          | 73                 |
| OSU-03012/3        | 3 $\mu$ M           | Celecoxib derivatives  | 37                 |
| UCN-01             | 33 nM               | 7-hydroxy staurosporine analog, Phase I/II studies available | 55,74              |
| BX-795, -912, -320 | 11–30 nM            | Aminopyridines   | 75                 |

Abbreviations: COX-2, cyclooxygenase-2; PDK, phosphoinositide-dependent kinase.

**Table 2** Select Akt inhibitors by class

| Name  | IC <sub>50</sub>                          | Comments   | Selected citations |
|---|---|--|--------------------|
| <i>ATP competitive inhibitors</i>                         |   |  |                    |
| Balanol analogs   | 4–5 nM                                    | Rationally designed  | 76                 |
| H-89  | 2.5 $\mu$ M                               | Protein kinase A inhibitor   | 77                 |
| NL-71-101   | 3.7 $\mu$ M                               | Developed from H-89  | 78                 |
| <i>Lipid-based/phosphatidylinositol analog inhibitors</i> |   |  |                    |
| PIA 5/6/23/24/25  | <5 $\mu$ M                                | Ether lipid analogs, prevent translocation of Akt  | 79–81              |
| Perifosine  | 5 $\mu$ M                                 | Prevents Akt translocation, phase II data available                                      | 50                 |
| PX-316  | 1.7 $\mu$ M                               | Binds to PH domain of Akt  | 82                 |
| PX-866  | 16.8 nM                                   | Inhibits PI signaling  |                    |
| <i>Pseudosubstrate Inhibitors</i>                         |   |  |                    |
| AKTide-2T   | 12 $\mu$ M                                |  | 83,84              |
| FOXO3 hybrid  | 1.1 $\mu$ M                               | Hybrid with AKTide-2T  | 30                 |
| FOXO3 hybrid modification                                 | 0.11 $\mu$ M                              | Replaced Ser w Ala   | 30                 |
| <i>Allosteric inhibitors of AKT kinase domain</i>         |   |  |                    |
| Compound 12   | AKT1 = 4.6 $\mu$ M<br>AKT2 = >250 $\mu$ M | First isozyme specific AKT inhibitor   | 85,86              |
| Compound 13   | AKT1 = 2.1 $\mu$ M<br>AKT2 = 21 $\mu$ M   | Dual activity  | 85,86              |
| Compounds 14-29   |   | Iterative improvements with greater specificity  | 85,86              |
| <i>Akt antibodies</i>                                     |   |  |                    |
| GST-anti-Akt1-MTS   |   | Cell-permeable antibody, blocks catalytic site   | 87                 |
| <i>Interaction with PH domain of AKT</i>                  |   |  |                    |
| Triciribine/API-2   |   | May interact with PH domain (?) Prior phase II trials at high doses showed high toxicity | 88,89              |
| TCN-P   |   | Triciribine monophosphate  | 90                 |
| Akt-in  |   | Synthesized peptide  | 84                 |
| <i>Unknown/multiple mechanism(s)</i>                      |   |  |                    |
| KP372-1   |   |  | 91                 |
| N10-substituted phenoxazines                              | 1–2 $\mu$ M                               | May bind ATP-binding site or act as allosteric inhibitors                                | 92                 |
| Genistein   |   | Inhibits multiple intracellular kinases  | 56,59,63           |

Abbreviation: PH, pleckstrin homology.

### Perifosine

Phospholipid analogues have been in use as medications for some time. Miltefosine demonstrated activity against many cancers and is still approved in Europe for use in cutaneous lymphoma and cutaneous breast cancer metastases. High rates of gastrointestinal toxicity and low bioavailability led to efforts to discover further modifications of phospholipid analogues with enhanced pharmaceutical potential.

Perifosine is a substituted alkylphosphocholine with oral bioavailability. In preclinical experiments, it causes cell-cycle arrest in G<sub>1</sub>/S or G<sub>2</sub>/M, probably through effects mediated by p21 upregulation.<sup>49</sup> These effects appear to occur through inhibition of Akt activation, although the mechanism is incompletely understood. As a phospholipid analogue, perifosine incorporates into the cell wall where it prevents Akt phosphorylation in a PDK1-independent manner.<sup>50</sup> This apparently involves interference with the normal association of PH domains with 3-PI moieties.

Phase I trials established the tolerability of perifosine with most frequent side effects being nausea, vomiting and diarrhea.<sup>51,52</sup> Two recent phase II trials of perifosine in CaP have been reported. A California Cancer

Consortium trial in 25 patients with biochemical recurrence following definitive therapy demonstrated biological and chemical activity, with 23% of patients having a decrease in PSA, although none were >50%.<sup>53</sup> A National Cancer Institute study of 19 men with metastatic AI CaP and average PSA of 180 ng/ml showed no objective or PSA responses, and four patients with PSA stabilization for 12 weeks.<sup>54</sup>

Although perifosine is apparently relatively ineffective as monotherapy, the strong preclinical rationale combined with preclinical results showing synergism with other Akt inhibitory drugs suggest the need for more trials examining its role in an adjunctive setting.<sup>55</sup> Phase I trials are already underway for perifosine combined with docetaxel, paclitaxel, gemcitabine and radiation therapy. Future phase II trials at our institution will examine the combination of perifosine with other inhibitors of the Akt pathway in CaP.

### Genistein

Genistein is a naturally occurring isoflavone found in soy-based products. Gastric and intestinal hydrolytic reactions convert it to a well-absorbed aglyconic form. In

addition, some Asian forms of fermented soy, such as miso, natto and tempeh, are rich in isoflavone aglycones. Speculation regarding the CaP-inhibitory effects of soy isoflavones exists because of significant epidemiologic differences in CaP incidence mirroring the consumption of, among other things, soy products.<sup>56</sup> Preclinical data demonstrate truly remarkable biochemical characteristics of genistein. In many different cell lines and xenografts, the tyrosine kinase inhibitory characteristics of genistein induce apoptosis, cell-cycle arrest, hinders proliferation, prevents angiogenesis, and blocks androgen and estrogen-stimulated transcription.<sup>56–60</sup> In addition, studies demonstrate genistein may oppose many cellular survival mechanisms induced by radiation, chemotherapy or androgen deprivation, suggesting the usefulness of this agent as adjunctive therapy.<sup>61,62</sup>

Subsequent analysis of these favorable biochemical changes reveal that many of them are mediated through Akt pathways (see Figure 1). For example, NF $\kappa$ B, an upstream regulator of the apoptotic Bcl-2 family and the inhibitors of cyclin dependent kinases, p21 and p27, is activated by many cellular stimuli. Li and Sarkar<sup>63</sup> demonstrated that NF $\kappa$ B activation in PC3 cells is mediated by Akt. In prostate cell lines, experiments at our institution confirm that genistein inhibits activation of Akt, thereby inducing apoptosis similar to other inhibitors of Akt (see Figure 2).<sup>64</sup> Inhibitors such as LY294002, although completely blocking Akt phosphorylation through PI3K inhibition, are too toxic for human use. Genistein may provide a non-toxic alternative while maintaining sufficient activity to effect a clinical response.

Some of the cellular effects of genistein seen *in vitro* cannot be initiated at attainable concentrations in humans, even when consuming large amounts of soy. Concentrated aglycone-rich food supplements, such as genistein combined polysaccharide (GCP), may allow higher plasma levels of isoflavones.<sup>58</sup> Recently, a prospective randomized study by Rannikko *et al.*<sup>65</sup> found that prostate tissue concentrates phytoestrogens includ-

ing genistein, thus suggesting that plasma concentrations underestimate tissue concentrations by over 50%.

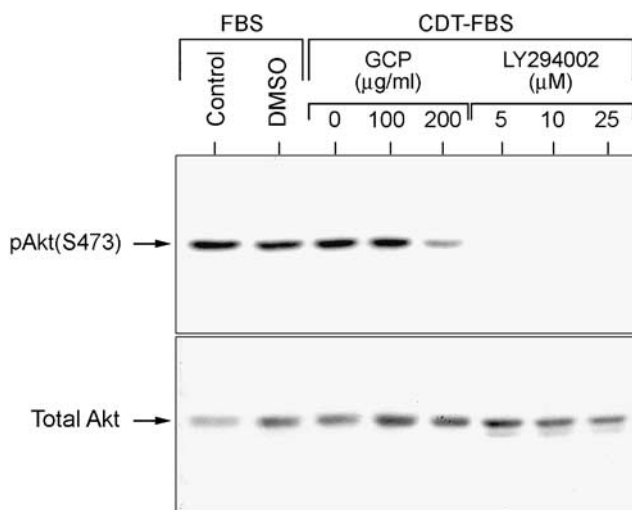
Given the promising preclinical data, studies are ongoing examining the activity of genistein *in vivo* against CaP. Phase I studies demonstrate general tolerability.<sup>66</sup> A phase II pilot study at our institution examined the ability of GCP to decrease PSA in men with histologically proven CaP. Sixty-two patients with elevated PSA were enrolled. Patients were categorized by prior treatment. Nine, seventeen and six had received prior prostatectomy, radiation therapy, or both, respectively. Fourteen were receiving intermittent hormone ablation and were currently off-cycle. Finally, 16 patients were on an active surveillance protocol. All patients received GCP supplements to be taken orally three times daily for 6 months. Three patients discontinued because of grade 2 diarrhea and seven patients discontinued for personal reasons. Of the 52 remaining patients, 8 had PSA reductions and 1 of them greater than 50%. Interestingly, all of these patients were in the watchful waiting subgroup. In analysis of the responding patients vs others, there did not appear to be any correlation with Gleason score. In addition, estrogenic effects of genistein were probably not the causative factor as testosterone was increased in five responders, decreased in one, and unchanged in two.<sup>67</sup>

On the basis of these results, further randomized studies are currently ongoing at our institution. In addition to examination of GCP as monotherapy in patients on watchful waiting protocols, we plan to study GCP in conjunction with androgen deprivation therapy in patients with biochemical relapse following definitive therapy.

## Patient selection

Biologic therapies modifying cellular signaling pathways generally show modest responses in most cases. This suggests the need to test such therapies in the adjunctive setting, possibly increasing the response to radiation, chemotherapy or androgen deprivation.<sup>3</sup> For ethical reasons, many existing studies will use Akt inhibitors in conjunction with docetaxel-based chemotherapy. This is a rational strategy as chemotherapy upregulates survival pathways that involve Akt activity.<sup>68,69</sup>

Further efforts to predict patient response to biologic agents by pretreatment measurements of activated pathways through direct tissue or serum analysis may allow individualized treatment. One method involves profiling the entire proteome in patients' serum using matrix assisted laser desorption/ionization-time-of-flight mass spectrometry. Multiple bioinformatics analyses of CaP patient serum and normal controls allows for proteomic 'fingerprints' with high discriminatory power. Once such profiles are produced, patients on clinical studies may be compared, allowing characterization of treatment effects on proteome profiles. Examination of the entire proteome may allow more efficient sorting of possible tumor markers. Eventually, exact identification of identified tumor markers is carried out by 2-D electrophoresis or liquid chromatography.<sup>70,71</sup> In the future, biopsy samples analyzed for expression of such markers may allow *ex vivo* modeling of diseased pathways. Subsequent study



**Figure 2** Western blot demonstrating the inhibition of Akt phosphorylation in the presence of GCP compared to LY294002 (Courtesy of Dr Clifford Tepper). GCP, genistein combined polysaccharide; DMSO, dimethylsulfoxide.

will eventually lead to individualized and targeted biologic therapy.

## Conclusions

Akt inhibition is a rational therapy in CaP treatment. Upregulation of this pathway is involved in initial neoplastic changes in some patients. An increasing number of patients show overexpression or overactivity of Akt as metastatic and AI diseases develop. Preclinical studies demonstrate the importance of this pathway in CaP and the possibility of targeting this pathway with any of an increasing number of inhibitors. In addition to preventing activation by blocking upstream signaling, strategies include allosteric inhibition, small molecule competitive inhibitors, pseudosubstrate inhibitors and others with multiple or unknown activity.

Clinical studies with agents known to act through Akt inhibition show some promise. Further studies examining vertical inhibition strategies to block Akt pathways more completely should be performed. More importantly, efforts to extend the activity of current therapeutic options through combination with Akt inhibitors and more accurate methods to select those patients most likely to benefit from Akt inhibition are needed.

## Acknowledgements

PNL is supported in part by the Tom and Melissa Egan Cancer Research Fund, Veterans' Administration of Northern California, RO1-CA120469 and NIH NO1 CM17101.

## References

- 1 Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C *et al*. Cancer statistics, 2006. *CA Cancer J Clin* 2006; **56**: 106–130.
- 2 Huggins C, Hodges C. Studies on prostate cancer: I. The effect of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* 1941; **1**: 293–297.
- 3 Nelson EC, Cambio AJ, Yang JC, Lara Jr PN, Evans CP. Biologic agents as adjunctive therapy prostate cancer: a rationale for use with androgen deprivation. *Nat Clin Pract Urol* 2007; **4**: 82–94.
- 4 Staal SP, Hartley JW, Rowe WP. Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. *Proc Natl Acad Sci USA* 1977; **74**: 3065–3067.
- 5 Li L, Ittmann MM, Ayala G, Tsai MJ, Amato RJ, Wheeler TM *et al*. The emerging role of the PI3-K-Akt pathway in prostate cancer progression. *Prostate Cancer Prostatic Dis* 2005; **8**: 108–118.
- 6 Coffey PJ, Jin J, Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* 1998; **335** (Part 1): 1–13.
- 7 Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 2002; **14**: 381–395.
- 8 Mulholland DJ, Dedhar S, Wu H, Nelson CC. PTEN and GSK3beta: key regulators of progression to androgen-independent prostate cancer. *Oncogene* 2006; **25**: 329–337.
- 9 Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci USA* 2000; **97**: 10832–10837.
- 10 Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y *et al*. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; **91**: 231–241.

- 11 Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* 2001; **20**: 7779–7786.
- 12 Majewski N, Nogueira V, Robey RB, Hay N. Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases. *Mol Cell Biol* 2004; **24**: 730–740.
- 13 Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K *et al*. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999; **399**: 597–601.
- 14 Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999; **399**: 601–605.
- 15 Papandreou CN, Logothetis CJ. Bortezomib as a potential treatment for prostate cancer. *Cancer Res* 2004; **64**: 5036–5043.
- 16 Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of MDM2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci USA* 2001; **98**: 11598–11603.
- 17 Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH *et al*. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* 2000; **60**: 6841–6845.
- 18 Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc Natl Acad Sci USA* 2001; **98**: 7200–7205.
- 19 Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY *et al*. Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J Biol Chem* 2003; **278**: 50902–50907.
- 20 Edwards J, Krishna NS, Witton CJ, Bartlett JM. Gene amplifications associated with the development of hormone-resistant prostate cancer. *Clin Cancer Res* 2003; **9**: 5271–5281.
- 21 Rubin MA, Gerstein A, Reid K, Bostwick DG, Cheng L, Parsons R *et al*. 10q23.3 loss of heterozygosity is higher in lymph node-positive (pT2-3,N+) versus lymph node-negative (pT2-3,N0) prostate cancer. *Hum Pathol* 2000; **31**: 504–508.
- 22 Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J *et al*. Prostate-specific deletion of the murine PTEN tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 2003; **4**: 209–221.
- 23 Majumder PK, Sellers WR. Akt-regulated pathways in prostate cancer. *Oncogene* 2005; **24**: 7465–7474.
- 24 Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. *Endocrinology* 2001; **142**: 4795–4805.
- 25 Kreisberg JI, Malik SN, Prihoda TJ, Bedolla RG, Troyer DA, Kreisberg S *et al*. Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer Res* 2004; **64**: 5232–5236.
- 26 Nelson EC, Cambio AJ, Yang JC, Ok J-H, Lara Jr PN, Evans CP. Clinical implications of neuroendocrine differentiation in prostate cancer. *Prostate Cancer Prostatic Dis* 2007; **10**: 6–14.
- 27 Granville CA, Memmott RM, Gills JJ, Dennis PA. Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. *Clin Cancer Res* 2006; **12**: 679–689.
- 28 Kim D, Cheng GZ, Lindsley CW, Yang H, Cheng JQ. Targeting the phosphatidylinositol-3 kinase/Akt pathway for the treatment of cancer. *Curr Opin Investig Drugs* 2005; **6**: 1250–1258.
- 29 Chen YL, Law PY, Loh HH. Inhibition of PI3K/Akt signaling: an emerging paradigm for targeted cancer therapy. *Curr Med Chem Anticancer Agents* 2005; **5**: 575–589.
- 30 Barnett SF, Bilodeau MT, Lindsley CW. The Akt/PKB family of protein kinases: a review of small molecule inhibitors and progress towards target validation. *Curr Top Med Chem* 2005; **5**: 109–125.
- 31 Stauffer F, Holzer P, Garcia-Echeverria C. Blocking the PI3K/PKB pathway in tumor cells. *Curr Med Chem Anticancer Agents* 2005; **5**: 449–462.

- 32 Kim YH, Lee YJ. TRAIL apoptosis is enhanced by quercetin through Akt dephosphorylation. *J Cell Biochem* 2007; **100**: 998–1009.
- 33 Xiao D, Li M, Herman-Antosiewicz A, Antosiewicz J, Xiao H, Lew KL *et al*. Diallyl trisulfide inhibits angiogenic features of human umbilical vein endothelial cells by causing Akt inactivation and down-regulation of VEGF and VEGF-R2. *Nutr Cancer* 2006; **55**: 94–107.
- 34 Deeb D, Jiang H, Gao X, Al-Holou S, Danyluk AL, Dulchavsky SA *et al*. Curcumin (diferuloyl-methane) sensitizes human prostate cancer cells to TRAIL/Apo2L-induced apoptosis by suppressing NF- $\kappa$ B via inhibition of pro-survival Akt signaling pathway. *J Pharmacol Exp Ther* 2007 [E-pub ahead of print].
- 35 Agarwal R, Agarwal C, Ichikawa H, Singh RP, Aggarwal BB *et al*. Anticancer potential of silymarin: from bench to bed side. *Anticancer Res* 2006; **26**: 4457–4498.
- 36 Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J Biol Chem* 2000; **275**: 11397–11403.
- 37 Zhu J, Huang JW, Tseng PH, Yang YT, Fowble J, Shiau CW *et al*. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. *Cancer Res* 2004; **64**: 4309–4318.
- 38 Ding H, Han C, Zhu J, Chen CS, D'Ambrosio SM. Celecoxib derivatives induce apoptosis via the disruption of mitochondrial membrane potential and activation of caspase 9. *Int J Cancer* 2005; **113**: 803–810.
- 39 Zha S, Gage WR, Sauvageot J, Saria EA, Putzi MJ, Ewing CM *et al*. Cyclooxygenase-2 is up-regulated in proliferative inflammatory atrophy of the prostate, but not in prostate carcinoma. *Cancer Res* 2001; **61**: 8617–8623.
- 40 O'Neill GP, Ford-Hutchinson AW. Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. *FEBS Lett* 1993; **330**: 156–160.
- 41 Kune GA, Kune S, Watson LF. Colorectal cancer risk, chronic illnesses, operations, and medications: case-control results from the Melbourne Colorectal Cancer Study. *Cancer Res* 1988; **48**: 4399–4404.
- 42 Kismet K, Akay MT, Abbasoglu O, Ercan A. Celecoxib: a potent cyclooxygenase-2 inhibitor in cancer prevention. *Cancer Detect Prev* 2004; **28**: 127–142.
- 43 Basler JW, Piazza GA. Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 selective inhibitors for prostate cancer chemoprevention. *J Urol* 2004; **171**: S59–S62; discussion S53–S62.
- 44 Pruthi RS, Derksen JE, Moore D, Carson CC, Grigson G, Watkins C *et al*. Phase II trial of celecoxib in prostate-specific antigen recurrent prostate cancer after definitive radiation therapy or radical prostatectomy. *Clin Cancer Res* 2006; **12**: 2172–2177.
- 45 Smith MR, Manola J, Kaufman DS, Oh WK, Bubley GJ, Kantoff PW. Celecoxib versus placebo for men with prostate cancer and a rising serum prostate-specific antigen after radical prostatectomy and/or radiation therapy. *J Clin Oncol* 2006; **24**: 2723–2728.
- 46 Kasimis B, Cogswell J, Hwang S, Chang VT, Srinivas S, Zhong F *et al*. High dose celecoxib(C) and docetaxel(D) in patients(pts)with hormone resistant prostate cancer(HRPC). Results of an ongoing phase II trial. *J Clin Oncol* 2005; **23S**, Abstract 4704.
- 47 Kattan JG, Bachour M, Farhat F, El Seoudi M, Younes F, Ghosn M *et al*. Phase II trial of weekly docetaxel, zoledronate and celecoxib for androgen-independent prostate cancer patients. *Proc Prostate Cancer Symp* 2006; Abstract 362.
- 48 Sooriakumaran P, Macanas-Pirard P, Fox S, Coley H, Bucca G, Lovell D *et al*. A blinded, randomized controlled trial of neo-adjuvant celecoxib in patients with early prostate cancer. *J Clin Oncol* 2006; **24S**, Abstract 4563.
- 49 Patel V, Lahusen T, Sy T, Sausville EA, Gutkind JS, Senderowicz AM. Perifosine, a novel alkylphospholipid, induces p21(WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest. *Cancer Res* 2002; **62**: 1401–1409.
- 50 Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. *Mol Cancer Ther* 2003; **2**: 1093–1103.
- 51 Crul M, Rosing H, de Klerk GJ, Dubbelman R, Traiser M, Reichert S *et al*. Phase I and pharmacological study of daily oral administration of perifosine (D-21266) in patients with advanced solid tumours. *Eur J Cancer* 2002; **38**: 1615–1621.
- 52 Van Ummersen L, Binger K, Volkman J, Marnocha R, Tutsch K, Kolesar J *et al*. A phase I trial of perifosine (NSC 639966) on a loading dose/maintenance dose schedule in patients with advanced cancer. *Clin Cancer Res* 2004; **10**: 7450–7456.
- 53 Chee KG, Lara PN, Longmate J, Twardowski P, Quinn DI, Chatta G *et al*. The AKT inhibitor perifosine in biochemically recurrent, hormone-sensitive prostate cancer (HSPC): a phase II California Cancer Consortium Trial. *J Clin Oncol* 2005; **23S**, Abstract 4563.
- 54 Posadas EM, Gulley J, Arlen PM, Trout A, Parnes HL, Wright J *et al*. A phase II study of perifosine in androgen independent prostate cancer. *Cancer Biol Ther* 2005; **4**: 1133–1137.
- 55 Dasmahapatra GP, Didolkar P, Alley MC, Ghosh S, Sausville EA, Roy KK. *In vitro* combination treatment with perifosine and UCN-01 demonstrates synergism against prostate (PC-3) and lung (A549) epithelial adenocarcinoma cell lines. *Clin Cancer Res* 2004; **10**: 5242–5252.
- 56 Sarkar FH, Adsule S, Padhye S, Kulkarni S, Li Y. The role of genistein and synthetic derivatives of isoflavone in cancer prevention and therapy. *Mini Rev Med Chem* 2006; **6**: 401–407.
- 57 Sarkar FH, Li Y. Mechanisms of cancer chemoprevention by soy isoflavone genistein. *Cancer Metastasis Rev* 2002; **21**: 265–280.
- 58 Bemis DL, Capodice JL, Desai M, Buttyan R, Katz AE. A concentrated aglycone isoflavone preparation (GCP) that demonstrates potent anti-prostate cancer activity *in vitro* and *in vivo*. *Clin Cancer Res* 2004; **10**: 5282–5292.
- 59 Bektic J, Guggenberger R, Eder IE, Pelzer AE, Berger AP, Bartsch G *et al*. Molecular effects of the isoflavonoid genistein in prostate cancer. *Clin Prostate Cancer* 2005; **4**: 124–129.
- 60 Skogseth H, Follstad T, Larsson E, Halgunset J. Transcription levels of invasion-related genes in prostate cancer cells are modified by inhibitors of tyrosine kinase. *APMIS* 2006; **114**: 364–371.
- 61 Chen HL, Holland WS, Lara PN, Gandara D, White RD, Gumerlock P. Preclinical study of genistein combined polysaccharide (GCP) and docetaxel (Doc) treatment of prostate cancer (CaP) cells. *J Clin Oncol* 2005; **23S**, Abstract 4711.
- 62 Holland WS, Shih D, Harse R, Vijaykumar S, Hackman R, Gandara D *et al*. Inhibition of the AKT pathway with genistein combined polysaccharide (GCP) plus external beam radiation therapy (EBRT) in prostate cancer (CaP) xenograft. *J Clin Oncol* 2005; **23S**, Abstract 3135.
- 63 Li Y, Sarkar FH. Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via Akt signaling pathway. *Clin Cancer Res* 2002; **8**: 2369–2377.
- 64 Tepper CG, Vinall RL, Wee CB, Xue L, Shi XB, Burich R *et al*. GCP-mediated growth inhibition and apoptosis of prostate cancer cells via androgen receptor-dependent and -independent mechanisms. *Prostate* 2007; **67**: 521–535.
- 65 Rannikko A, Petas A, Rannikko S, Adlercreutz H. Plasma and prostate phytoestrogen concentrations in prostate cancer patients after oral phytoestrogen supplementation. *Prostate* 2006; **66**: 82–87.
- 66 Poisson BA, Takimoto C, Shapiro A, Gallot L, Nabhan C, Lieberman R *et al*. Pharmacokinetic analysis of the putative prostate cancer chemopreventive agent, genistein. *Proc Am Soc Clin Oncol* 2001; **20**, Abstract 334.
- 67 deVere White RW, Hackman RM, Soares SE, Beckett LA, Li Y, Sun B. Effects of a genistein-rich extract on PSA levels in men with a history of prostate cancer. *Urology* 2004; **63**: 259–263.
- 68 Chuang SE, Yeh PY, Lu YS, Lai GM, Liao CM, Gao M *et al*. Basal levels and patterns of anticancer drug-induced activation of nuclear factor-kappaB (NF-kappaB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. *Biochem Pharmacol* 2002; **63**: 1709–1716.

- 69 Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. *Cancer Res* 2001; **61**: 3986–3997.
- 70 Kennedy S. Proteomic profiling from human samples: the body fluid alternative. *Toxicol Lett* 2001; **120**: 379–384.
- 71 Rabilloud T. Detecting proteins separated by 2-D gel electrophoresis. *Anal Chem* 2000; **72**: 48A–55A.
- 72 Patel MI, Subbaramaiah K, Du B, Chang M, Yang P, Newman RA et al. Celecoxib inhibits prostate cancer growth: evidence of a cyclooxygenase-2-independent mechanism. *Clin Cancer Res* 2005; **11**: 1999–2007.
- 73 Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH et al. 3-phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. *Cancer Res* 2004; **64**: 1444–1451.
- 74 Sato S, Fujita N, Tsuruo T. Interference with PDK1-Akt survival signaling pathway by UCN-01 (7-hydroxystaurosporine). *Oncogene* 2002; **21**: 1727–1738.
- 75 Feldman RI, Wu JM, Polokoff MA, Kochanny MJ, Dinter H, Zhu D et al. Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. *J Biol Chem* 2005; **280**: 19867–19874.
- 76 Breitenlechner CB, Wegge T, Berillon L, Graul K, Marzenell K, Friebe WG et al. Structure-based optimization of novel azepane derivatives as PKB inhibitors. *J Med Chem* 2004; **47**: 1375–1390.
- 77 Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T et al. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* 1990; **265**: 5267–5272.
- 78 Reuveni H, Livnah N, Geiger T, Klein S, Ohne O, Cohen I et al. Toward a PKB inhibitor: modification of a selective PKA inhibitor by rational design. *Biochemistry* 2002; **41**: 10304–10314.
- 79 Hu Y, Qiao L, Wang S, Rong SB, Meuillet EJ, Berggren M et al. 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J Med Chem* 2000; **43**: 3045–3051.
- 80 Castillo SS, Brognard J, Petukhov PA, Zhang C, Tsurutani J, Granville CA et al. Preferential inhibition of Akt and killing of Akt-dependent cancer cells by rationally designed phosphatidylinositol ether lipid analogues. *Cancer Res* 2004; **64**: 2782–2792.
- 81 Gills JJ, Holbeck S, Hollingshead M, Hewitt SM, Kozikowski AP, Dennis PA. Spectrum of activity and molecular correlates of response to phosphatidylinositol ether lipid analogues, novel lipid-based inhibitors of Akt. *Mol Cancer Ther* 2006; **5**: 713–722.
- 82 Meuillet EJ, Ihle N, Baker AF, Gard JM, Stamper C, Williams R et al. *In vivo* molecular pharmacology and antitumor activity of the targeted Akt inhibitor PX-316. *Oncol Res* 2004; **14**: 513–527.
- 83 Luo Y, Smith RA, Guan R, Liu X, Klinghofer V, Shen J et al. Pseudosubstrate peptides inhibit Akt and induce cell growth inhibition. *Biochemistry* 2004; **43**: 1254–1263.
- 84 Hiromura M, Okada F, Obata T, Auguin D, Shibata T, Roumestand C et al. Inhibition of Akt kinase activity by a peptide spanning the betaA strand of the proto-oncogene TCL1. *J Biol Chem* 2004; **279**: 53407–53418.
- 85 Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE et al. Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochem J* 2005; **385**: 399–408.
- 86 Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D et al. Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorg Med Chem Lett* 2005; **15**: 761–764.
- 87 Shin I, Edl J, Biswas S, Lin PC, Mernaugh R, Arteaga CL. Proapoptotic activity of cell-permeable anti-Akt single-chain antibodies. *Cancer Res* 2005; **65**: 2815–2824.
- 88 Hoffman K, Holmes FA, Fraschini G, Esparza L, Frye D, Raber MN et al. Phase I-II study: tricyclic nucleoside phosphate for metastatic breast cancer. *Cancer Chemother Pharmacol* 1996; **37**: 254–258.
- 89 Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI et al. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res* 2004; **64**: 4394–4399.
- 90 Feun LG, Blessing JA, Barrett RJ, Hanjani P. A phase II trial of tricyclic nucleoside phosphate in patients with advanced squamous cell carcinoma of the cervix. A Gynecologic Oncology Group Study. *Am J Clin Oncol* 1993; **16**: 506–508.
- 91 Mandal M, Kim S, Younes MN, Jasser SA, El-Naggar AK, Mills GB et al. The Akt inhibitor KP372-1 suppresses Akt activity and cell proliferation and induces apoptosis in thyroid cancer cells. *Br J Cancer* 2005; **92**: 1899–1905.
- 92 Thimmaiah KN, Easton JB, Germain GS, Morton CL, Kamath S, Buolamwini JK et al. Identification of N10-substituted phenoxazines as potent and specific inhibitors of Akt signaling. *J Biol Chem* 2005; **280**: 31924–31935.

# Prostate cancer and markers of bone metabolism: diagnostic, prognostic, and therapeutic implications

Eric C. Nelson · Christopher P. Evans ·  
Chong-Xian Pan · Primo N. Lara Jr

Received: 1 February 2007 / Accepted: 15 May 2007 / Published online: 12 June 2007  
© Springer-Verlag 2007

**Abstract** Knowledge of bone metastases complicating advanced prostate cancer (CaP) is increasingly relevant in patient selection for novel therapies. Current nuclear bone scintigraphy imaging has limited specificity for prostate metastases. As serum bone markers do correlate with bony lesions, they may play multiple roles in patients with advanced CaP. Currently, these markers play a role in prognostic nomograms for CaP. Recent studies suggest an expanding role for bone markers in the diagnosis and selection of patients for novel therapies. In the future, therapeutic roles for some of these marker pathways will emerge, eventually allowing greater individualization of patient care.

**Keywords** Androgen-independent prostate cancer · Bone markers · Bone metastases · Osteoprotegerin · Prostate cancer

## Introduction

In advanced prostate cancer (CaP), metastatic deposits in bone are common and are the most frequent source of pain and morbidity [1]. “Skeletal related events” or SREs include vertebral compression fractures, complete vertebral collapse, spinal cord or spinal nerve entrapment, pathologic fractures, bone pain, and significant serum calcium abnormalities. These may be directly related to metastatic deposits in the bone, or be secondary to medical or surgical castration, which forms the foundation of current treatment strategies for metastatic CaP [2]. Osteoporosis due to castration is the more common cause, with only 7–16% of fractures being caused directly by metastatic lesions [3]. In addition, an association exists between the diagnosis of CaP and baseline osteopenia and osteoporosis prior to treatment or the development of metastatic disease [4]. Overall, yearly incidence of SREs is about 12% in patients with metastatic androgen-independent CaP [5].

## Bone metabolism overview

Normal bone metabolism is distinguished by two opposing activities, which are coupled in both space and time and subject to tight control. The formation of new bone by osteoblasts and the resorption of old bone by osteoclasts are both constitutively active and the balance of these activities ultimately determines bone mass.

Bony metastatic lesions in CaP are classically thought of as osteoblastic or sclerotic lesions caused by a relative increase in bone formation. Though this is true for the majority of lesions, recent studies have indicated a therapeutic role for osteoclast inhibition in the prevention of SREs. Specifically, zoledronic acid has been shown to be the only

---

E. C. Nelson · C. P. Evans  
Department of Urology, University of California,  
Davis Medical Center, 4860 Y St, Suite 3500,  
Sacramento, CA 95817, USA  
e-mail: enelson06m@gmail.com

C. P. Evans  
e-mail: christopher.evans@ucdmc.ucdavis.edu

C.-X. Pan · P. N. Lara Jr (✉)  
Department of Internal Medicine,  
Division of Hematology and Oncology,  
School of Medicine, University of California,  
Davis, 4501 X Street, Sacramento, CA 95817, USA  
e-mail: primo.lara@ucdmc.ucdavis.edu

C.-X. Pan  
e-mail: chong-xian.pan@ucdmc.ucdavis.edu

effective bisphosphonate for decreasing SREs in patients with advanced CaP [6]. This activity by an osteoclast inhibitor in what are typically osteoblastic lesions emphasizes the close coupling of the action of these two cells.

Current diagnostic strategies for metastatic CaP in bone rely on nuclear bone scintigraphy scans. Unfortunately, these are expensive, require special equipment, are relatively cumbersome and expose the patient to radiation. As these scans detect increased bone formation, they are highly sensitive for detecting metastatic CaP lesions. However, they lack specificity and do not accurately measure treatment response as increased bone formation may correlate with healing areas of lytic lesions [7]. The need for more specificity in diagnostic algorithms for CaP bone metastases suggests the possibility of using bone markers, either alone, or in combination with imaging studies to increase the accuracy of diagnosis.

Bone markers are typically biologically inactive peptide fragments or small proteins cleaved from larger proteins and released into the blood during the formation or resorption of bone. A list of bone markers used in CaP appears in Table 1. Only those with most clinical relevance will be discussed here.

**Table 1** Selected markers of bone metabolism relevant to CaP

|                               | Markers                                   | Measured in |
|-------------------------------|---|-------------|
| Markers of formation          | tALP                                      | Serum       |
|                               | bALP                                      | Serum       |
|                               | PINP/PIIINP                               | Serum       |
|                               | OC  | Serum       |
|                               | PICP                                      | Serum       |
| Markers of resorption         | BSP                                       | Serum       |
|                               | ICTP                                      | Serum       |
|                               | CTx                                       | Serum       |
|                               | NTx                                       | Serum       |
|                               | TRAP                                      | Serum       |
|                               | Deoxypyridinoline                         | Serum/Urine |
|                               | Pyridinoline                              | Serum/Urine |
|                               | Hydroxyproline                            | Urine       |
|                               | N-terminal telopeptide of type I collagen | Serum/Urine |
|                               | Calcium:Creatinine ratio                  | Urine       |
| Markers of osteoclastogenesis | OPG                                       | Serum       |
|                               | RANKL                                     | Serum       |

*bALP* Bone alkaline phosphatase; *BSP* bone sialoprotein; *CaP* prostate cancer; *CTx* carboxy-terminal telopeptide; *ICTP* pyridinoline cross-linked carboxy-terminal telopeptide; *NTx* amino-terminal telopeptide; *OC* osteocalcin; *OPG* osteoprotegerin; *PICP* procollagen I carboxy-terminal propeptide; *PIIINP* procollagen III amino-terminal propeptide; *PINP* procollagen I amino-terminal propeptide; *RANKL* receptor activator of NF $\kappa$ B ligand; *tALP* total alkaline phosphatase; *TRAP* tartrate resistant acid phosphatase

## Formation markers

Among the markers of bone formation are alkaline phosphatase, procollagen peptide fragments (which are cleaved at the time of bone collagen formation), and osteocalcin (OC). Total alkaline phosphatase (tALP) has also traditionally been used as a marker for bone formation because of its wide availability as part of the comprehensive metabolic panel. However, it lacks true specificity for bone due to its presence in biliary, hepatic, and intestinal tissues. The bone isoenzyme form of alkaline phosphatase (bALP), a tetrameric protein located in the plasma membrane of osteoblasts, has been used to overcome this deficiency. The procollagen peptide fragments, on the other hand, are by-products of the extracellular cleavage of pro-collagen during bone formation and are specific for bone formation. These fragments are released into the circulation and are detected clinically using antibodies directed against the amino- (PINP) and carboxy- (PICP) terminal ends of procollagen I, or against the amino-terminal end of procollagen III (PIIINP) [8]. Finally, OC is a vitamin K-dependent, non-collagenous low molecular weight protein produced and released by osteoblasts. OC, also known as Gla protein, is widely deposited in the bony matrix and is the most abundant organic component of bone after collagen. Its presence in human sera is believed to be an index of osteoblastic activity and has been proposed as a useful marker for treatment response in metastatic CaP [9].

## Resorption markers

Among the markers for bone resorption are the pyridinium compounds pyridinoline and deoxypyridinoline, both of which are found in Type I collagen and are amino acid derivatives. It has long been known that the tensile strength of bone is largely due to cross-linking of these derivatives in Type I collagen [10]. The deoxypyridinoline crosslink is felt to be specific to bone since it is only found in Type I collagen. On the other hand, pyridinoline is also present in Type II collagen, a component of articular cartilage. During bone resorption, these pyridinium cross-links are released into the circulation. Once released by the resorptive process, the pyridinium cross-links are not metabolized further; therefore, they represent degradative end products of mature collagen [11]. Another resorption marker is the collagen degradative product telopeptide, on which pyridinium cross-links are attached [12]. These include the amino- or carboxy-terminal telopeptide assay called N-telopeptide (NTx) and C-telopeptide (CTx) fragments, respectively. The commercial assay employs an antibody against the alpha-2 chain of Type I bone collagen fibrils. A related marker of a slightly larger telopeptide of the carboxy terminal is abbreviated ICTP [13]. Though some of these

markers may also be measured in urine, serum collection allows simultaneous measurement of other markers, is convenient in the setting of clinical trials, and may be more reproducible.

#### Osteoclastogenesis markers

A new class of bone markers may more closely reflect the biochemistry of the metastatic site by giving insights into cellular signaling. Osteoprotegerin (OPG), receptor activator of NF $\kappa$ B (RANK), and receptor activator of NF $\kappa$ B ligand (RANKL) are thought to belong to an important cytokine system controlling the process of osteoclastogenesis. Preclinical data confirms the importance of the relative concentration of these cellular signaling molecules in controlling the balance of bone formation and breakdown [14]. These markers may not only serve as a surrogate for osteoclastic activity but may prove to be important in future therapeutic interventions.

#### Bone markers: potential diagnostic applications

Because nearly all the bone markers discussed above show strong correlation with bone scan results in multiple studies, the use of bone markers as diagnostic instruments has been proposed. Various studies have shown promising results for ICTP [15], PINP [16, 17], CTx [17] and OPG [18]. Most studied markers demonstrate a wide range of results, which negatively impacts sensitivity and specificity. In addition, other conditions such as arthritis or bone healing secondary to trauma may further decrease specificity. Therefore, though mean bone marker concentration is of diagnostic significance for a population, individual patient counseling is difficult.

As mentioned previously, bone scintigraphy scans have high sensitivity but low specificity. Any effort to increase the specificity of diagnostic testing requires a highly specific “gold standard” for comparison. In this setting, only biopsy of each metastatic deposit would qualify as such a gold standard and this is obviously not feasible. Increase in lesion size on imaging of untreated patients may provide a surrogate marker but is also rather unhelpful, as most patients will be treated. Lesion shrinkage may not be seen on imaging with treatment as bone scans cannot discriminate between bone formation activity of metastatic disease and the healing process following response to treatment.

The strong rationale supporting bone markers as a diagnostic modality encourages efforts to find a surrogate marker verifying adequate specificity. One strategy is to acquire an ability to predict future metastatic deposits on imaging. If bone markers are able to do this, it may provide the needed proof of concept. At least one small study has demonstrated that increases in bone markers precede evi-

dence of bony metastatic lesions on bone scan by about 3 months [19]. This study measured urinary pyridinolines normalized by creatinine and should be repeated using other markers to confirm the concept. In the future, algorithms using bone scintigraphy in combination with prostate specific antigen (PSA) and various bone markers will maximize sensitivity and specificity in the diagnosis of CaP.

#### Bone markers: prognostic implications

Compared to proving diagnostic specificity, it is easier to demonstrate the ability of bone markers to stratify patients into prognostic categories. The use of bone markers in prognostic nomograms is well known. At least since 1992, tALP has been included in many nomograms predicting outcomes for different patient populations with CaP [20–23]. As discussed above, other bone markers demonstrate the ability to predict future bone metastases. This suggests they might have a prognostic role in predicting overall survival.

Correlative studies confirm the prognostic capabilities of some bone metabolism markers. Brown et al. [24] retrospectively examined bALP and NTx in large patient cohorts from phase III studies examining zoledronic acid in metastatic CaP and other neoplasms [25, 26]. In patients in the placebo arms of the trials, both NTx and bALP were statistically significant predictors of outcome, though NTx was superior [24]. Because of the results of the prospective phase III trial, zoledronic acid treatment is likely to be given to many patients with metastatic hormone refractory CaP [25]. As zoledronic acid significantly affects NTx levels, a second study by Cook et al. [27] examined all CaP patients using only baseline NTx and bALP levels. They found both markers were significantly associated with overall survival and progression-free survival, but only bALP was independently associated with overall survival on multivariate analysis. Dividing patients into quartiles based on bALP correlated strongly with overall survival and thus may be a useful prognostic tool for clinicians and patients.

Our group prospectively evaluated the prognostic and predictive significance of selected markers of bone metabolism in the context of a randomized phase II clinical trial of a matrix metalloproteinase inhibitor in hormone refractory CaP [28]. Markers of bone formation (OC, PINP and PIIINP) and resorption (NTx, pyridinoline and deoxypyridinoline) in serum were measured using commercial enzyme immunoassays. Marker values were dichotomized at the median and correlated with overall survival and progression-free survival by log-rank testing. Of eighty patients enrolled, 69 had evaluable baseline serum specimens.

We found that lower levels of tALP, NTx, deoxypyridinoline, OC, PINP, and PIIINP were all significant predictors of improved median and progression-free survival. In our study population, the significant prognostic value of these markers was not affected by bisphosphonate treatment. On multivariate analysis, log PINP and PIIINP remained significant as part of a model including hemoglobin and log PSA [29].

These retrospective and prospective clinical results should be prospectively validated in the phase III setting. Toward that end, we have initiated a molecular correlative study of the prognostic and possible predictive value of bone markers in patients with bone-metastatic hormone refractory CaP in conjunction with Southwest Oncology Group trial S0421. This phase III, placebo-controlled trial of docetaxel/prednisone with or without Atrasentan will randomize 706 patients with hormone refractory CaP and bone metastases, and provides an ideal setting to study bone markers. Earlier trials indicate Atrasentan's modest therapeutic effect is most significant in patients with bone metastases [30]. Serial measurements of bone markers will hopefully allow creation of validated prognostic nomograms. In addition, discovery of bone marker parameters predicting a beneficial response to Atrasentan will in the future allow appropriate patient selection for therapy.

In addition to predicting survival, bone markers may be able to predict response to treatment with androgen ablation. As discussed below, studies show a clear correlation of bone markers with treatment response on imaging and clinical improvement. With further refinement, new markers more closely reflecting actual bone biochemistry may predict not only overall survival for patient populations, but may allow for individualized treatment.

#### *Bone markers: disease activity monitoring*

As early as 1992, studies have examined the ability of bone markers to monitor the metastatic CaP response to androgen deprivation [9]. Clear correlation of bone marker changes on serial measurements with clinical response, whether based on imaging or PSA, has been conclusively demonstrated. Urinary pyridinolines [19], PICP [31], ICTP [15] and PINP [32] have all shown significant correlations. In addition, Koizumi et al suggest that the ratios of markers of bone formation may be helpful in following response to treatment [32].

In the future, bone markers may play a role in monitoring disease in patients with or without clear metastatic disease on bone scan. Though further studies are needed before this occurs, a strong rationale exists for using bone markers in this setting. As mentioned above, elevations in bone markers may precede evidence of bony metastatic lesions by up to three months. Thus, the usefulness of these

markers for monitoring disease is suggested, even in patients with no corroborating imaging.

#### *Bone markers: therapeutic*

The novel bone marker OPG, in contrast to most other markers, is a biologically active member of the tumor necrosis factor (TNF) superfamily [33]. As a decoy receptor for RANKL, it inhibits downstream signaling activating osteoclasts [14]. It may prevent apoptosis by inhibiting TNF related apoptosis inducing ligand or TRAIL [34]. Pre-clinical studies also suggest a role for OPG in promoting angiogenesis [35]. The variety of effects apparently related to this cytokine system suggest it not only provides a marker of diagnosis and prognosis, but may suggest therapeutic interventions [14, 34, 35].

A fully human monoclonal antibody against RANKL has recently entered clinical trials. Denosumab may be considered an OPG analogue in that it binds and inactivates RANKL. A phase III randomized, double blind, multicenter comparison of denosumab with the current reference standard, zoledronic acid, in patients with metastatic CaP began in April 2006. Results are eagerly anticipated.

The superiority of OPG as a marker, discussed below, may be due to the fact that it represents not only a byproduct of bone metabolism, but also an active participant in the microenvironment affecting metastatic growth and related bone turnover.

#### *Bone marker comparisons*

Few head to head comparisons of the various bone markers have been performed and those studies listed in Table 2 may sometimes yield contradictory results. This may be due to different patient populations and the fact that different bone markers measure different stages of bone metabolism and are affected differently by androgen deprivation [13, 16]. Nevertheless, some conclusions can be drawn. tALP and bALP have been the most studied and consistently demonstrate equivalent, and sometimes superior prognostic value compared to other markers [13, 27]. Of the other markers of bone formation, PINP may be preferable to use as a marker [16, 17, 29]. OC is clearly inferior to other markers [5, 13, 18].

Deciding which of the markers of bone resorption is most accurate is more difficult. One study comparing multiple bone markers suggests that OPG is most helpful for prognostic use [18]. As a marker of osteoclastogenesis, it may serve as a marker for osteoclast action while giving additional prognostic information based on tumor biochemistry. However, OPG is not, strictly speaking, a marker of bone resorption. Future studies should prospectively assess OPG in comparison to CTx, NTx, ICTP, and the pyridinolines.

**Table 2** Selected clinical studies of bone markers in prostate cancer

| Year of publication | Markers  | Conclusions   | Reference |
|---------------------|--|---|-----------|
| 1992                | <b>OC</b>  | No prognostic significance to baseline levels, questionable significant to changes with treatment   | [9]       |
| 1996                | <b>Urinary pyridinoline, urinary deoxypyridinoline, tALP, OC</b>   | Urine markers correlate with response to treatment and predicted new lesions on bone scan   | [19]      |
| 1997                | <b>PICP, ICTP</b>  | PICP correlated more closely with bone scan, but both decrease with treatment   | [31]      |
| 1999                | <b>ICTP</b>  | ICTP effective diagnostic tool. Serial measurements useful for following disease  | [15]      |
| 1999                | bALP, ICTP, serum Calcium, parathyroid hormone, urinary Calcium:Creatinine ratio, urinary deoxypyridinoline                    | Resorption markers correlate best with pain scores  | [36]      |
| 2000                | tALP, urinary pyridoline/deoxypyridoline, urinary hydroxyproline, urine Calcium:Creatinine ratio, PICP, bone Gla protein, ICTP | Deoxypyridoline can predict SREs with high specificity. tALP as good as bALP because liver metastases are rare  | [5]       |
| 2001                | bALP, OC, PICP, ICTP, CTx, NTx, pyridinoline, deoxypyridinoline  | Castration caused significant changes in bALP, NTx, CTx, and the pyridinolines. For initial diagnosis in the hormone-naïve, bALP, and deoxypyridinoline were best. Following hormone therapy, bALP and ICTP were superior | [13]      |
| 2001                | PINP, PICP, bALP, OC, ICTP   | All markers were higher in patients with metastases except OC. PINP correlated best with disease extent   | [16]      |
| 2002                | <b>PINP, bALP, OC, ratios of all three</b>   | Ratios of markers may be useful   | [32]      |
| 2003                | BALP, PINP, urinary NTx, urinary CTx, serum CTx, ICTP  | PINP and serum CTx demonstrate 100% sensitivity and specificity   | [17]      |
| 2004                | OPG  | OPG may be effective for following disease  | [34]      |
| 2004                | tALP, bALP, PINP, OC, BSP, CTx, NTx, TRAP, RANKL, OPG  | OPG was an independent predictor of CaP death   | [18]      |
| 2005                | Urinary NTx, tALP  | Both markers predict outcomes on univariate, but not multivariate analysis  | [37]      |
| 2005                | OC, PINP, PIIINP, NTx, pyridinoline, deoxypyridinoline   | Baseline levels of PINP and PIIINP were independent predictors of survival. Bisphosphonate use did not affect this  | [29]      |
| 2005                | Urinary NTx, bALP  | NTx effective for predicting SREs and disease progression in patients on zoledronic acid  | [38]      |
| 2005                | Urinary NTx, bALP  | NTx better than bALP for predicting disease progression in patients on placebo  | [24]      |
| 2006                | Urinary NTx, bALP  | bALP independent predictor of outcomes, better than NTx in CaP  | [27]      |

Markers in bold indicate serial measurements were performed

*bALP* Bone alkaline phosphatase; *BSP* bone sialoprotein; *CaP* prostate cancer; *CTx* carboxy-terminal telopeptide; *ICTP* pyridinoline cross-linked carboxy-terminal telopeptide; *NTx* amino-terminal telopeptide; *OC* osteocalcin; *OPG* osteoprotegerin; *PICP* procollagen I carboxy-terminal propeptide; *PIIINP* procollagen III amino-terminal propeptide; *PINP* procollagen I amino-terminal propeptide; *RANKL* receptor activator of NF- $\kappa$ B ligand; *tALP* total alkaline phosphatase; *TRAP* tartrate resistant acid phosphatase

## Conclusions

Markers of bone metabolism play a role in current prognostic nomograms for CaP. In the future, their role will increase, especially in the areas of earlier diagnosis of bony metastatic disease and the monitoring of therapeutic interventions. Bone markers will extend the specificity of current diagnostic imaging techniques and panels of bone markers may reach sufficient accuracy to be used alone. Serial measurements of bone markers will allow greater insight into the action of novel therapies currently under development for CaP. Finally, bone markers of metabolic activity, such as OPG, will suggest future strategies that will allow individualization of oncologic therapy.

**Acknowledgments** PNL is supported in part by RO1-CA120469, Veterans' Administration of Northern California, and NIH NO1 CM17101.

## References

1. Michaelson MD, Smith MR (2005) Bisphosphonates for treatment and prevention of bone metastases. *J Clin Oncol* 23(32):8219–8224
2. Smith MR (2006) Markers of bone metabolism in prostate cancer. *Cancer Treat Rev* 1 (32 Suppl):23–26
3. Shahinian VB, Kuo YF, Freeman JL, Goodwin JS (2005) Risk of fracture after androgen deprivation for prostate cancer. *N Engl J Med* 352(2):154–164
4. Hussain SA, Weston R, Stephenson RN, George E, Parr NJ (2003) Immediate dual energy X-ray absorptiometry reveals a high incidence of osteoporosis in patients with advanced prostate cancer before hormonal manipulation. *BJU Int* 92(7):690–694
5. Berruti A, Dogliotti L, Bitossi R, Fasolis G, Gorzegno G, Bellina M et al (2000) Incidence of skeletal complications in patients with bone metastatic prostate cancer and hormone refractory disease: predictive role of bone resorption and formation markers evaluated at baseline. *J Urol* 164(4):1248–1253
6. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L et al (2004) Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. *J Natl Cancer Inst* 96(11):879–882
7. Fontana A, Delmas PD (2000) Markers of bone turnover in bone metastases. *Cancer* 88(12 Suppl):2952–2960
8. Suvanto-Luukkonen E, Risteli L, Sundstrom H, Penttinen J, Kaupila A, Risteli J (1997) Comparison of three serum assays for bone collagen formation during postmenopausal estrogen-progestin therapy. *Clin Chim Acta* 266(2):105–116
9. Arai Y, Takeuchi H, Oishi K, Yoshida O (1992) Osteocalcin: is it a useful marker of bone metastasis and response to treatment in advanced prostate cancer? *Prostate* 20(3):169–177
10. Hanson DA, Eyre DR (1996) Molecular site specificity of pyridinoline and pyrrole cross-links in type I collagen of human bone. *J Biol Chem* 271(43):26508–26516
11. Demers LM, Costa L, Lipton A (2000) Biochemical markers and skeletal metastases. *Cancer* 88(12 Suppl):2919–2926
12. Hanson DA, Weis MA, Bollen AM, Maslan SL, Singer FR, Eyre DR (1992) A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. *J Bone Miner Res* 7(11):1251–1258
13. Tamada T, Sone T, Tomomitsu T, Jo Y, Tanaka H, Fukunaga M (2001) Biochemical markers for the detection of bone metastasis in patients with prostate cancer: diagnostic efficacy and the effect of hormonal therapy. *J Bone Miner Metab* 19(1):45–51
14. Hofbauer LC, Neubauer A, Heufelder AE (2001) Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. *Cancer* 92(3):460–470
15. Koga H, Naito S, Koto S, Sakamoto N, Nakashima M, Yamasaki T et al (1999) Use of bone turnover marker, pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP), in the assessment and monitoring of bone metastasis in prostate cancer. *Prostate* 39(1):1–7
16. Koizumi M, Yonese J, Fukui I, Ogata E (2001) The serum level of the amino-terminal propeptide of type I procollagen is a sensitive marker for prostate cancer metastasis to bone. *BJU Int* 87(4):348–351
17. de la Piedra C, Castro-Errecaborde NA, Traba ML, Mendez-Davila C, Garcia-Moreno C, Rodriguez de Acuna L et al (2003) Bone remodeling markers in the detection of bone metastases in prostate cancer. *Clin Chim Acta* 331(1–2):45–53
18. Jung K, Lein M, Stephan C, Von Hosslin K, Semjonow A, Sinha P et al (2004) Comparison of 10 serum bone turnover markers in prostate carcinoma patients with bone metastatic spread: diagnostic and prognostic implications. *Int J Cancer* 111(5):783–791
19. Takeuchi S, Arai K, Saitoh H, Yoshida K, Miura M (1996) Urinary pyridinoline and deoxypyridinoline as potential markers of bone metastasis in patients with prostate cancer. *J Urol* 156(5):1691–1695
20. Petrylak DP, Scher HI, Li Z, Myers CE, Geller NL (1992) Prognostic factors for survival of patients with bidimensionally measurable metastatic hormone-refractory prostatic cancer treated with single-agent chemotherapy. *Cancer* 70(12):2870–2878
21. Kelly WK, Scher HI, Mazumdar M, Vlamis V, Schwartz M, Fossa SD (1993) Prostate-specific antigen as a measure of disease outcome in metastatic hormone-refractory prostate cancer. *J Clin Oncol* 11(4):607–615
22. Smaletz O, Scher HI, Small EJ, Verbel DA, McMillan A, Regan K et al (2002) Nomogram for overall survival of patients with progressive metastatic prostate cancer after castration. *J Clin Oncol* 20(19):3972–3982
23. Halabi S, Small EJ, Kantoff PW, Kattan MW, Kaplan EB, Dawson NA et al (2003) Prognostic model for predicting survival in men with hormone-refractory metastatic prostate cancer. *J Clin Oncol* 21(7):1232–1237
24. Brown JE, Cook RJ, Major P, Lipton A, Saad F, Smith M et al (2005) Bone turnover markers as predictors of skeletal complications in prostate cancer, lung cancer, and other solid tumors. *J Natl Cancer Inst* 97(1):59–69
25. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L et al (2002) A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. *J Natl Cancer Inst* 94(19):1458–1468
26. Rosen LS, Gordon D, Tchekmedyian S, Yanagihara R, Hirsh V, Krzakowski M et al (2003) Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial—the Zoledronic acid lung cancer and other solid tumors study group. *J Clin Oncol* 21(16):3150–3157
27. Cook RJ, Coleman R, Brown J, Lipton A, Major P, Hei YJ et al (2006) Markers of bone metabolism and survival in men with hormone-refractory metastatic prostate cancer. *Clin Cancer Res* 12(11 Pt 1):3361–3367
28. Lara PN Jr, Stadler WM, Longmate J, Quinn DI, Wexler J, Van Loan M et al (2006) A randomized phase II trial of the matrix metalloproteinase inhibitor BMS-275291 in hormone-refractory

- prostate cancer patients with bone metastases. *Clin Cancer Res* 12(5):1556–1563
29. Lara PN, Longmate J, Stadler W, van Loan M, Wexler J, Quinn DI et al (2005) Markers of bone metabolism predict survival in hormone refractory prostate cancer (HRPC): results from a randomized California Cancer Consortium and Univ of Chicago trial. *J Clin Oncol* 23S(16S) Abst 4569
  30. Nelson JB (2005) Endothelin receptor antagonists. *World J Urol* 23(1):19–27
  31. Yoshida K, Sumi S, Arai K, Koga F, Umeda H, Hosoya Y et al (1997) Serum concentration of type I collagen metabolites as a quantitative marker of bone metastases in patients with prostate carcinoma. *Cancer* 80(9):1760–1767
  32. Koizumi M, Yonese J, Fukui I, Ogata E (2002) Metabolic gaps in bone formation may be a novel marker to monitor the osseous metastasis of prostate cancer. *J Urol* 167(4):1863–1866
  33. Holen I, Shipman CM (2006) Role of osteoprotegerin (OPG) in cancer. *Clin Sci (Lond)* 110(3):279–291
  34. Eaton CL, Wells JM, Holen I, Croucher PI, Hamdy FC (2004) Serum osteoprotegerin (OPG) levels are associated with disease progression and response to androgen ablation in patients with prostate cancer. *Prostate* 59(3):304–310
  35. Cross SS, Yang Z, Brown NJ, Balasubramanian SP, Evans CA, Woodward JK et al (2006) Osteoprotegerin (OPG)—a potential new role in the regulation of endothelial cell phenotype and tumour angiogenesis? *Int J Cancer* 118(8):1901–1908
  36. Berruti A, Dogliotti L, Gorzegno G, Torta M, Tampellini M, Tucci M et al (1999) Differential patterns of bone turnover in relation to bone pain and disease extent in bone in cancer patients with skeletal metastases. *Clin Chem* 45(8 Pt 1):1240–1247
  37. Berruti A, Tucci M, Mosca A, Tarabuzzi R, Gorzegno G, Terrone C et al (2005) Predictive factors for skeletal complications in hormone-refractory prostate cancer patients with metastatic bone disease. *Br J Cancer* 93(6):633–638
  38. Coleman RE, Major P, Lipton A, Brown JE, Lee KA, Smith M et al (2005) Predictive value of bone resorption and formation markers in cancer patients with bone metastases receiving the bisphosphonate zoledronic acid. *J Clin Oncol* 23(22):4925–4935

## ORIGINAL ARTICLE

## Src family kinase oncogenic potential and pathways in prostate cancer as revealed by AZD0530

Y-M Chang<sup>1</sup>, L Bai<sup>1</sup>, S Liu<sup>2</sup>, JC Yang<sup>1</sup>, H-J Kung<sup>1,2,3</sup> and CP Evans<sup>1,3</sup><sup>1</sup>Department of Urology, University of California at Davis, Sacramento, CA, USA; <sup>2</sup>Department of Biological Chemistry and Molecular Medicine, University of California at Davis, Sacramento, CA, USA and <sup>3</sup>Cancer Center, University of California at Davis, Sacramento, CA, USA

Prostate cancer is the most frequently diagnosed cancer in American men. We have previously demonstrated that Src mediates androgen-independent proliferation in prostate cancer. We sought to investigate the Src-mediated oncogenic pathways and tumor biology using AZD0530, a novel Src family kinase/Abl dual-kinase inhibitor that is entering phase II clinical trials. We show that while both Src and Abl are expressed in all prostate cancer cell lines, Src but not Abl is activated in the prostate. Furthermore, Src activation is inhibited by AZD0530 in a rapid and dose-dependent manner. We show that Src mediates cell proliferation in DU145 and PC3 cells at the G1 phase of cell cycle. Src inhibition resulted in decreased binding of  $\beta$ -catenin to the promoters of G1 phase cell cycle regulators cyclin D1 and c-Myc. C-Myc may also be regulated at the protein level by extracellular signal-regulated kinase 1/2 and GSK3 $\beta$ . Cell motility factors focal adhesion kinase, p130CAS and paxillin activation in DU145 and PC3 cells were also inhibited. Administration of AZD0530 in mice reduced orthotopic DU145 xenograft growth by 45%. We have further delineated the Src-mediated oncogenic growth and migration pathways in prostate cancer and established mechanistic rationale for Src inhibition as novel therapy in the treatment of prostate cancer.

*Oncogene* advance online publication, 4 August 2008; doi:10.1038/onc.2008.250

**Keywords:** Src; FAK; prostate cancer; AZD0530; proliferation; migration

## Introduction

Prostate cancer is the second leading cause of cancer death in men in the United States (Jemal *et al.*, 2008). Though prostate cancer growth is hormonally regulated, antiandrogen therapy inevitably results in disease

progression with uncontrolled growth and metastasis. An important mediator of this process is Src, a prototypical non-receptor tyrosine kinase (Lee *et al.*, 2001; Desai *et al.*, 2006).

The role of Src in human malignancies has not been fully appreciated in part because of the lack of frequent mutations in human cancers. Nevertheless, Src overexpression and activation are associated with numerous types of cancers (Biscardi *et al.*, 2000; Yeatman, 2004; Zhu *et al.*, 2007). Increasing evidence connects Src activity to prostate carcinogenesis. Src activity is required for androgen-independent activation of androgen receptor mediated by neuropeptide (Lee *et al.*, 2001; Desai *et al.*, 2006), epidermal growth factor (Guo *et al.*, 2006) and interleukin-8 (Lee *et al.*, 2004). Src and focal adhesion kinase (FAK), a Src substrate, are also involved in interleukin-8-induced migration of LNCaP. The application of pan-Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) leads to significant suppression of androgen-independent growth and migration of LNCaP (Lee *et al.*, 2001, 2004), as well as migration of PC3 and DU145 (Slack *et al.*, 2001). Dasatinib, a Src family kinase (SFK)/Abl dual-inhibitor, inhibits cell adhesion and migration of DU145 (Nam *et al.*, 2005b). Besides growth (Lee *et al.*, 2001, 2004; Kotha *et al.*, 2006), survival (Unni *et al.*, 2004; Nam *et al.*, 2005a; Kotha *et al.*, 2006) and metastasis (Lee *et al.*, 2004; Nam *et al.*, 2005b), Src is also implicated in angiogenesis (Gray *et al.*, 2005) and neuroendocrine differentiation (Bang *et al.*, 1994). Overall, these studies suggest that Src plays pleiotropic roles in prostate cancer, often in a cell context-dependent manner and that Src is a promising target for intervention.

Src is an integrator of divergent signals. In prostate cancer cells, Src is activated by growth factors, cytokines, chemokines and gastrin-releasing peptide. Src activation leads to the activation of FAK and Etk (endothelial/epithelial tyrosine kinase), kinases consistently activated or overexpressed in prostate cancer cells (Rovin *et al.*, 2002; Guo *et al.*, 2006). The pleiotropic effects of Src activity are almost certainly due to the multiple signal pathways engaged by Src and its accompanying kinases. Src is able to channel phosphorylation signals through Ras/Raf/extracellular signal-regulated kinase (ERK) 1/2 and in certain cells,

Correspondence: Dr CP Evans, Department of Urology, University of California-Davis Medical Center, 4860 Y St Suite 3500, Sacramento, CA 95817, USA. E-mail: cpevans@ucdavis.edu and Dr H-J Kung E-mail: hkung@ucdavis.edu

Received 20 February 2008; revised 3 June 2008; accepted 10 June 2008

phosphatidylinositol 3-kinase (PI3K)/AKT pathways. Somewhat selective to SFKs is its ability to activate signal and transducer of transcription (STAT) 3 and  $\beta$ -catenin, which leads to the activation of c-Myc (Bowman *et al.*, 2001; Furstoss *et al.*, 2002; Farkas *et al.*, 2005) and consequently cyclin D1 (Steiner *et al.*, 1998; Taj *et al.*, 2001; Devi *et al.*, 2002). Although STAT3 was shown to regulate cyclin D1 levels in prostate cancer, it remains unclear whether Src is an upstream regulator of STAT3 in prostate cancer cells, as some Src inhibitors do not diminish STAT3 activation and STAT3 can also be activated by Janus family kinases (Nam *et al.*, 2005b; Kotha *et al.*, 2006). The role of  $\beta$ -catenin activation in prostate carcinogenesis has been extensively documented (de la Taille *et al.*, 2003; Chen *et al.*, 2004; Cronauer *et al.*, 2005; Verras and Sun, 2006). The importance of c-Myc activation in prostate cancer is underscored by recent reports that c-Myc overexpression in mice prostate epithelial tissues gives rise to malignant lesions and that a significant fraction of prostate cancers amplify region 8q24 encompassing c-Myc (Bromann *et al.*, 2004; Dong, 2006). What is not well understood is the role of Src in the activation of  $\beta$ -catenin and c-Myc in prostate cancer. As for signaling pathways of migration and invasion in prostate cancer, it was shown that Src, through activated FAK, phosphorylates p130CAS (CRK-associated substrate) and upregulates matrix metalloproteinase (MMP)-9 (Hauck *et al.*, 2002; Planas-Silva *et al.*, 2006). In summary, Src transmits multiple signals including Ras/Raf/ERK1/2, PI3K/AKT,  $\beta$ -catenin/c-Myc/cyclin D1 and FAK/p130CAS/MMP-9 to induce growth, survival and migration in various types of cancer cells. Whether Src directly mediates cellular changes in prostate cancer through these signals remain unclear.

Recent interest in Src as a target for molecule-specific therapy has led to the development of small molecule inhibitors (Golas *et al.*, 2003; Lombardo *et al.*, 2004; Lee and Gautschi, 2006). Dasatinib inhibits PC3 growth (Lombardo *et al.*, 2004; Park *et al.*, 2008) and DU145 migration (Nam *et al.*, 2005b). Although Dasatinib is shown to inhibit growth *in vitro* in prostate cancer and has been suggested to inhibit proliferation through Lyn not Src, its mechanism of action in inhibiting cell proliferation remains unclear. AZD0530 (AstraZeneca, Alderley Park, UK), a 5-, 7-substituted anilinoquinazoline, is another novel SFK/Abl dual-inhibitor (Figure 1a) (Hennequin *et al.*, 2006; Lee and Gautschi, 2006). An oral compound with clinical therapeutic potential and low toxicity in phase I trials, it is highly specific with most kinases having *in vitro* kinase IC<sub>50</sub> values greater than 10  $\mu$ M. AZD0530 has antimigratory and modest antiproliferative effects *in vitro* in breast cancer (Hiscox *et al.*, 2006). AZD0530 has not, however, been previously studied in prostate cancer nor have Src-mediated signal pathways inhibited by this compound been defined. This study provides the first characterizations of the molecular and biological effects of AZD0530 in prostate cancer.

We show in this study that Src inhibition leads to growth suppression and cell cycle arrest in prostate cancer, which is accompanied by inactivation of ERK1/

2 and AKT, activation of GSK3 $\beta$  and downregulation of  $\beta$ -catenin, c-Myc and cyclin D1. Focal adhesion kinase and p130CAS phosphorylation are also attenuated as Src activity is inhibited, leading to significantly reduced cell migration. We also extended the analysis of AZD0530 as an antitumor agent *in vivo*. Using DU145 as our orthotopic mouse model, we show that AZD0530 is an inhibitor of growth *in vivo*. These studies provide important information regarding this small molecule inhibitor and set the stage for NCI approved phase II trials, using AZD0530 in advanced prostate cancer.

## Results

*Src is expressed and activated in prostate cancer cell lines*  
Autophosphorylation of Src at tyrosine 419 (Y419) is a surrogate marker of its activity (Bjelfman *et al.*, 1990). Src is expressed in LNCaP, DU145 and PC3 cell lines and increased Src activity correlates with more aggressive phenotypes (Bang *et al.*, 1994; Lee *et al.*, 2001, 2004; Nam *et al.*, 2005b; Kotha *et al.*, 2006). Src activity and expression levels in CWR22Rv1, LAPC-4 and immortalized normal prostate epithelial cell lines such as RWPE-1 and PZ-HPV7, however, have not previously been characterized. We therefore sought to compare and contrast the relative Src activation and expression levels in these cell lines. As Abl is also an AZD0530 target, we sought to characterize Abl in prostate cell lines as well.

Two Src isoforms were detected (Figure 1b) and were confirmed as Src through transfection experiments with wild-type Src cDNA constructs (data not shown). Src is expressed and activated in prostate cell lines. Notably, DU145 and PC3, cell lines with higher rates of proliferation and increased cell motility demonstrate an increased activated-to-total Src ratio when compared to other phenotypically less aggressive cell lines. Accounting for  $\alpha$ -tubulin levels, immortalized cells express more Src but have lower activated-to-total Src ratio than cancer cells. Although Abl is expressed in all prostate cell lines, it is not activated.

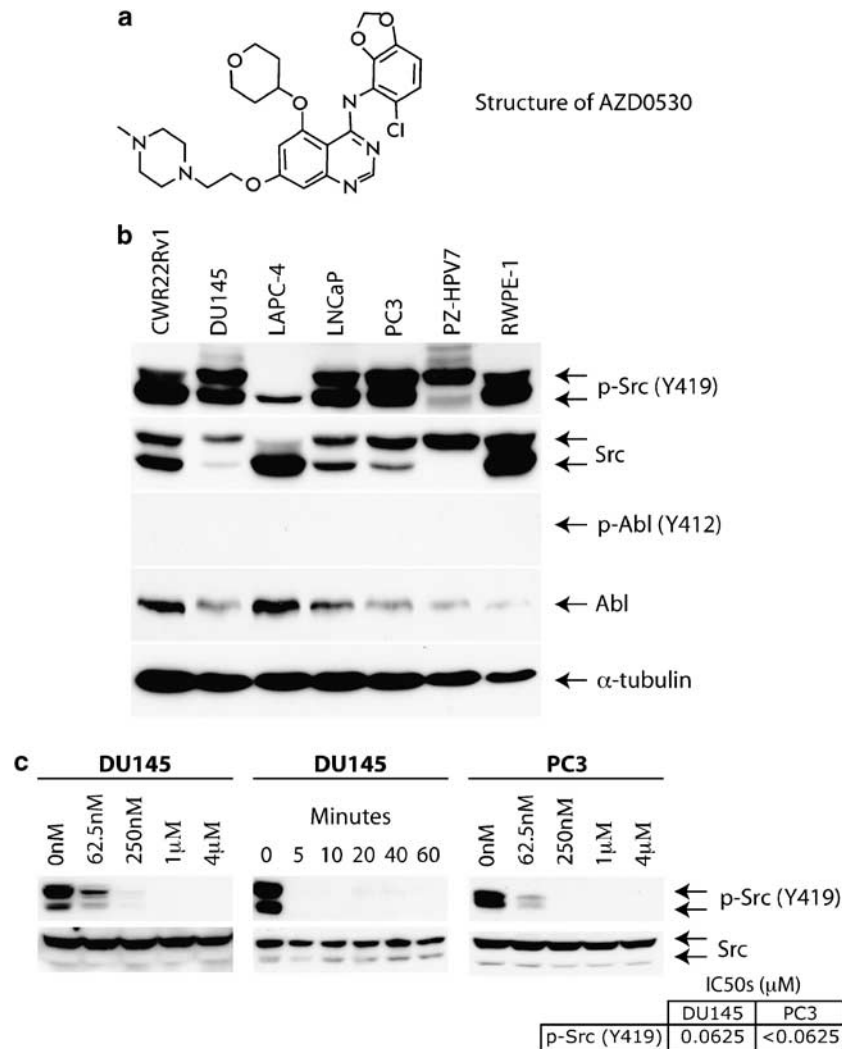
Our analysis with other SFKs suggests that Src is the predominant species expressed in prostate cancer cell lines (data not shown). As AZD0530 inhibits all SFKs at comparable concentrations, our data apply to all members. For simplicity, we will describe our results in the context of Src.

### *AZD0530 is a potent and rapid inhibitor of cellular Src activation*

We were interested in characterizing cellular Src inhibition by AZD0530. In DU145 and PC3, AZD0530 inhibited Src activation in a dose-dependent manner (Figure 1c, left, right). Src inhibition by AZD0530 was also rapid, within 5 min of treatment (Figure 1c, center).

### *AZD0530 inhibits growth and induces cell cycle arrest of prostate cancer*

Src is involved in prostate cancer cell proliferation. We were therefore interested in the effectiveness of



**Figure 1** AZD0530 inhibits Src activation through inhibition of Y419 phosphorylation. **(a)** The chemical structure of AZD0530. **(b)** Commonly used cell lines were harvested and probed with Abl, p-Abl, phospho-Src Y419 and Src antibodies demonstrating relative increased ratio of activated-to-total Src in DU145 and PC3 cells. **(c)** Src autophosphorylation in DU145 and PC3 cells were inhibited in a dose-dependent manner by AZD0530 following 30-min treatment (left and right, respectively) or rapid manner by 1 μM of AZD0530 (middle).

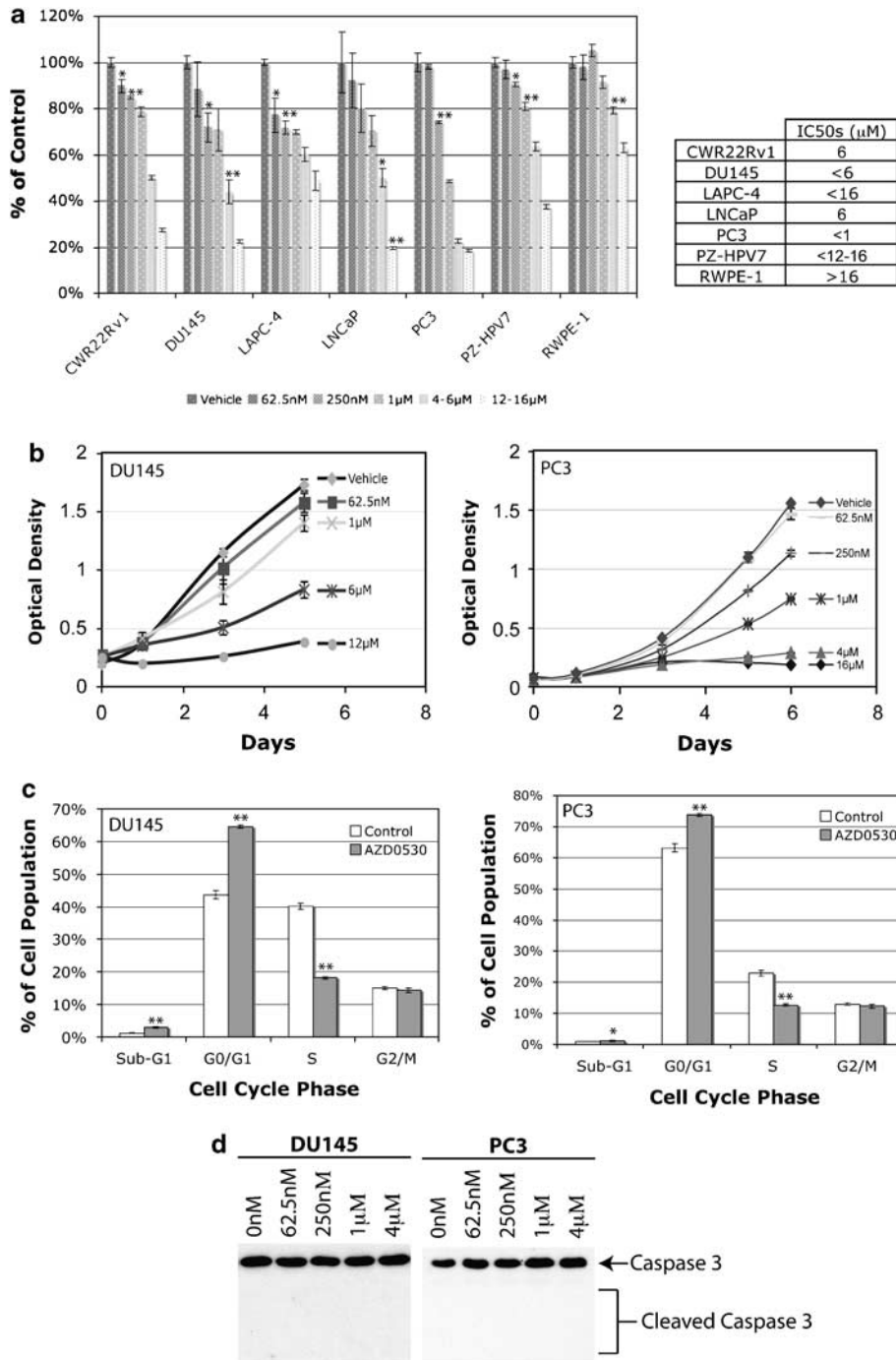
AZD0530 against mitogenesis. Single treatment with AZD0530 resulted in dose-dependent decrease of the number of cells in all cell lines (Figure 2a). LAPC-4, which has the smallest relative active-to-total Src ratio, is the most resistant against AZD0530 among prostate cancer cell lines. Immortalized nonmalignant cell lines PZ-HPV7 and RWPE-1 are also on average more resistant to Src inhibition than cancer cell lines. Figure 2b shows the kinetics and the dose-responses of growth inhibition for DU145 and PC3 cells. These analyses substantiate the growth inhibitory effects of AZD0530 on prostate cancer cells.

Although AZD0530 decreased the number of prostate cancer cells over time, it was unclear whether this is secondary to apoptosis or decreased cell proliferation. Studies of other Src inhibitors on DU145 induced apoptosis and cell cycle arrest at the G0/G1 phase of the cell cycle (Nam *et al.*, 2005a; Kotha *et al.*, 2006). We

thus sought to clarify the effects of AZD0530 using DU145 and PC3 as our models (Figure 2c). AZD0530 treatment of DU145 and PC3 respectively increased the proportion of G0/G1 cells by 21 and 11% and concurrently decreased S cells by 22 and 10% in the cell cycle, respectively. The fraction of apoptotic cells (sub-G1) is very low in both treated and untreated DU145 and PC3 samples. Furthermore, there is no significant caspase 3 cleavage following AZD0530 treatment (Figure 2d). Thus, the decreased numbers of cells is not due to apoptosis but to cell cycle arrest at G1/S.

#### Inhibition of c-Myc and cyclin D1 expression and downregulation of β-catenin by AZD0530

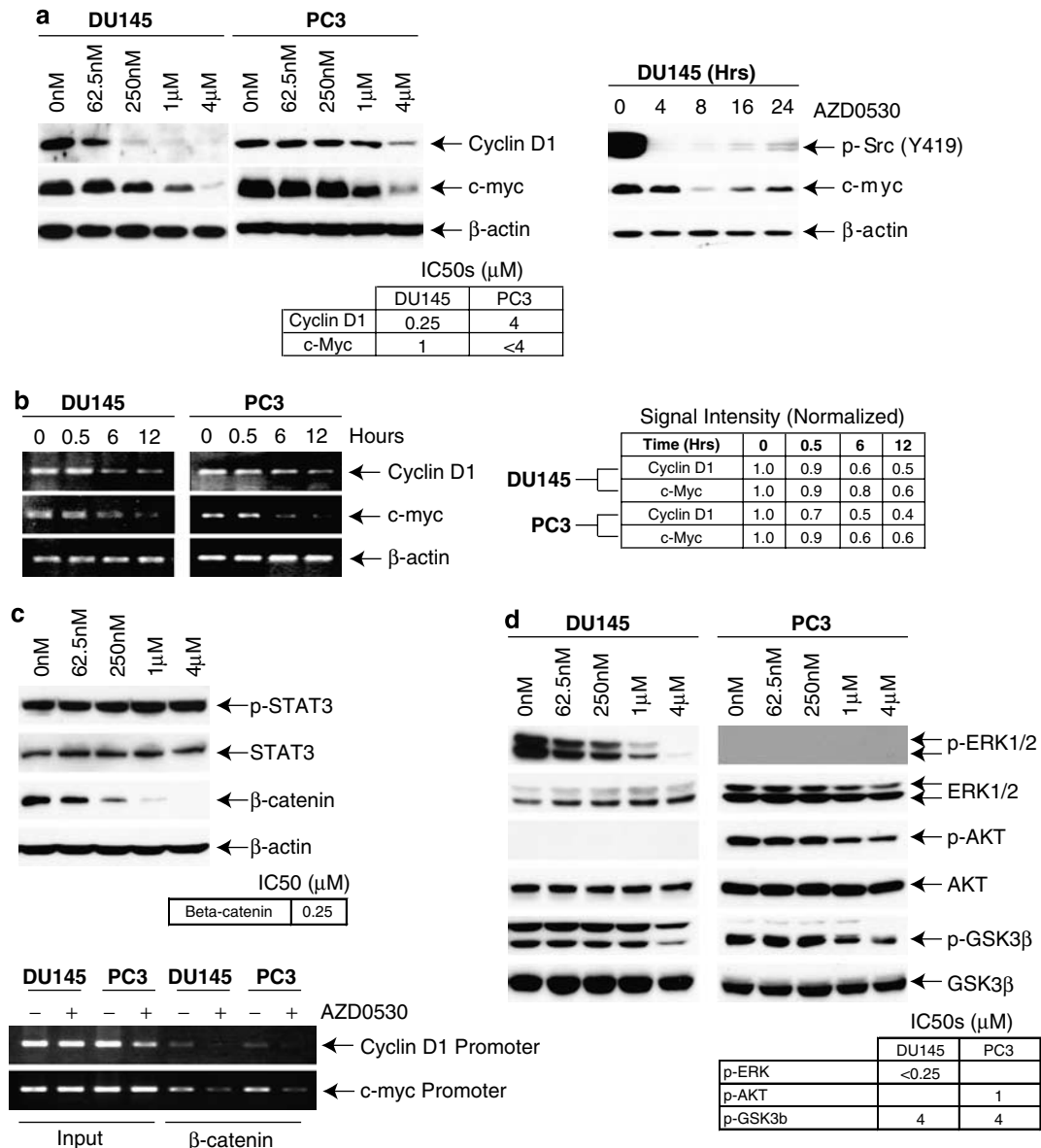
The effect of AZD0530 on G1/S transition prompted us to study its effect on c-Myc, an Src target gene



**Figure 2** AZD0530 inhibits cell proliferation at G0/G1-S transition. **(a)** Single administration of AZD0530 inhibited cell proliferation in a dose-dependent manner in 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay showing immortalized cells on average being more resistant than malignant cell lines. **(b)** AZD0530 inhibited DU145 and PC3 proliferation in a dose-dependent manner over time. **(c)** AZD0530 induced G1/S cell cycle arrest but not apoptosis in DU145 and PC3 cells. **(d)** AZD0530 did not induce apoptosis in DU145 and PC3 cells after 2 days, as shown by the lack of caspase 3 cleavage. Columns, mean; bars, standard error; \* $P < 0.05$  ( $n = 3$ ); \*\* $P < 0.01$  ( $n = 3$ ).

(Barone and Courtneidge, 1995) and cyclin D1, the rate limiting factor for cellular proliferation (Quelle *et al.*, 1993; Albanese *et al.*, 1995; Watanabe *et al.*, 1996). Both were downregulated upon AZD0530 treatment (Figure 3a). Since c-Myc is more resistant to AZD0530 than Src, we sought to examine the kinetics of AZD0530 on c-Myc. Although both c-Myc and phospho-Src

levels decrease after AZD0530 treatment, they both rebound over time, with relatively small changes in phospho-Src levels corresponding to larger changes seen in c-Myc. Cyclin D1 and c-Myc in DU145 cells are more sensitive to Src inhibition than PC3 cells. We further show corresponding reductions in transcripts (Figure 3b).



**Figure 3** AZD0530 inhibits cell proliferation through  $\beta$ -catenin, ERK1/2 and GSK3 $\beta$ -mediated cyclin D1 and c-myc regulation. (a) DU145 and PC3 cells treated with AZD0530 shows dose-dependent decreases in levels of cyclin D1 and c-Myc. Corresponding rebound of phospho-Src and c-Myc is seen over time. (b) Cyclin D1 and c-Myc transcript levels decreased following 4  $\mu$ M AZD0530 treatment. (c) AZD0530 treatment did not inhibit signal and transducer of transcription 3 activation after 30 min but downregulated  $\beta$ -catenin after 24 h (top). DU145 and PC3 treated with 4  $\mu$ M of AZD0530 for 12 h shows decreased binding of  $\beta$ -catenin to cyclin D1 and c-Myc promoter regions (bottom). (d) AZD0530 inhibited ERK1/2 and GSK3 $\beta$  phosphorylation in DU145 and AKT and GSK3 $\beta$  phosphorylation in PC3 after 30 min.

To study the upstream effectors that regulate c-Myc and cyclin D1 transcription, we noted that both STAT3 and  $\beta$ -catenin are Src targets and key transcriptional factors of c-Myc and cyclin D1 (Morin, 1999; Prathapam *et al.*, 2006). We previously showed that Src enhances STAT3 tyrosine phosphorylation through Etk (Tsai *et al.*, 2000). Other studies also showed that STAT3 mediates signals between Src and cyclin D1 in some prostate cancer cells (Gao *et al.*, 2005; Kotha *et al.*, 2006). Since PC3 does not express STAT3 (Yuan *et al.*, 2005), we focused on DU145. In DU145, AZD0530 treatment does not affect STAT3 phosphory-

lation, indicating its activation via a Src-independent pathway (Figure 3c, top).

We then turned our attention to  $\beta$ -catenin.  $\beta$ -catenin is a Src substrate (Roura *et al.*, 1999) and its synthesis is activated by Src (Karni *et al.*, 2005). It has also been shown to mediate both c-Myc and cyclin D1 transcription (Morin, 1999; Prathapam *et al.*, 2006). The protein level of  $\beta$ -catenin is highly sensitive to AZD0530 treatment (Figure 3c, top). Furthermore, AZD0530 treatment results in decreased binding of  $\beta$ -catenin to both cyclin D1 and c-Myc promoters (Figure 3c, bottom). These data taken together suggest that Src

mediates cell cycle progression by the induction of c-Myc and cyclin D1 transcription through increased  $\beta$ -catenin expression.

#### *Inhibition of ERK1/2 and GSK3 $\beta$ phosphorylation by AZD0530*

In addition to transcriptional regulation by  $\beta$ -catenin, c-Myc protein is regulated by ERK1/2 and AKT-GSK3 $\beta$ . GSK3 $\beta$  phosphorylation of c-Myc at T58 leads to ubiquitin-mediated proteosomal degradation, whereas ERK1/2-mediated phosphorylation at S62 stabilizes c-Myc (Dominguez-Sola and Dalla-Favera, 2004; Sears, 2004). GSK3 $\beta$  in turn is inactivated by AKT and ERK1/2 (Cross *et al.*, 1995; Cheng *et al.*, 2005; Kim *et al.*, 2007). Likewise, GSK3 $\beta$  negatively regulates the stability of  $\beta$ -catenin. We therefore wondered if AZD0530 inhibits ERK1/2 and AKT. ERK1/2 was inhibited by AZD0530 treatment, whereas AKT is constitutively inactive DU145 (Figure 3d). The lack of AKT activity is consistent with the presence of intact phosphatase and tensin homolog pathway in DU145, which diminishes PI3K-mediated AKT activation. This may also account for the lack of regulation of survival pathway by AZD0530 in DU145. ERK1/2 in PC3 cells on the other hand is not constitutively activated. AKT in PC3, however, is inhibited by AZD0530. AZD0530 treatment results in the removal of the inhibitory phosphorylation of GSK3 $\beta$  at S9 in both cell lines (Figure 3d) and therefore increased GSK3 $\beta$  activity, although at a higher concentration than that of  $\beta$ -catenin and ERK1/2 inhibition seen in DU145. These results suggest that in DU145 and PC3, ERK1/2 and AKT contribute to Src-

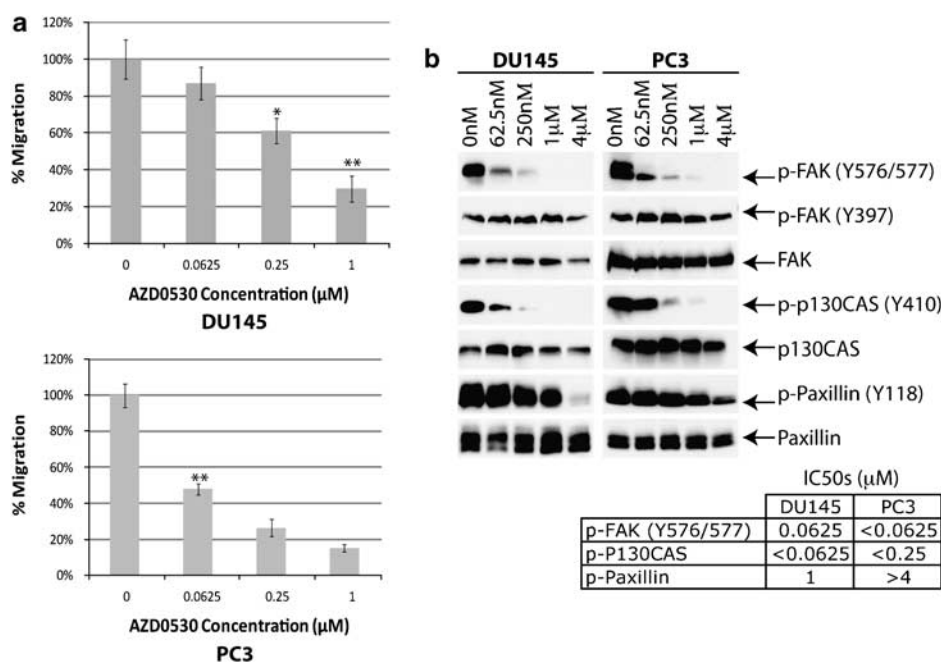
mediated stabilization of c-Myc, respectively. Furthermore, GSK3 $\beta$  is not responsible for Src-mediated stabilization of c-Myc and  $\beta$ -catenin in DU145.

#### *AZD0530 is an inhibitor of cell migration*

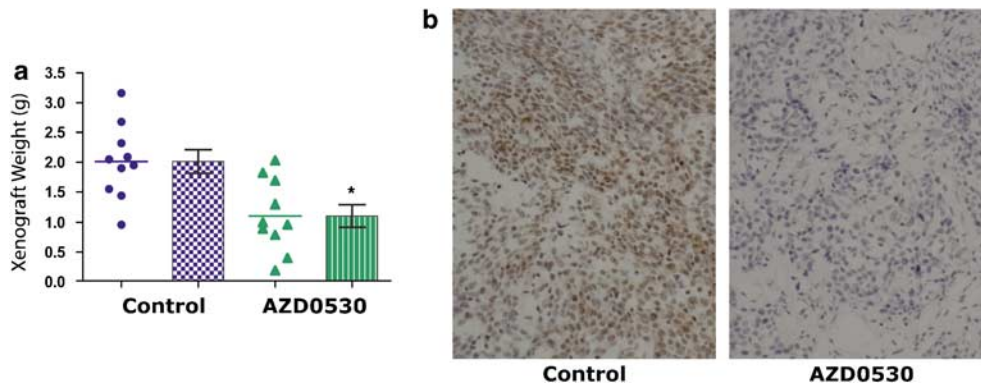
Src is an integral part of cell migration signaling pathway. We were therefore interested in whether AZD0530 effectively inhibits cell migration. We show that AZD0530 inhibits DU145 and PC3 migration in the Boyden chamber in a dose-dependent manner (Figure 4a).

Src and FAK are known to cross-activate, and enhanced migratory activity is linked to increased FAK expression and activation (Schaller, 2001; Slack *et al.*, 2001). Although autophosphorylation of FAK Y397 is necessary for its activity, Src phosphorylation of FAK Y576/Y577 is important in enhancing downstream signaling pathways (Parsons, 2003). AZD0530 treatment inhibited phosphorylation of Y576/577 but not Y397 (Figure 4b), indicating that AZD0530 targets Src but not FAK.

P130CAS is also an Src substrate involved in the formation of focal adhesion complexes. As shown in Figure 4b, p130CAS phosphorylation is inhibited by AZD0530. Furthermore, phosphorylation of paxillin, an adaptor protein and an Src-FAK substrate important in recruiting other proteins to the focal adhesion complex, is also inhibited by AZD0530, although at a higher of AZD0530 concentration than Src or FAK. This may reflect the fact that AZD0530 does not inhibit FAK autokinase activity (as reflected by the same level of Y397 phosphorylation), which continues to phosphorylate paxillin.



**Figure 4** AZD0530 inhibits cell migration through Src-mediated FAK activation. (a) DU145 (top) and PC3 (bottom) treated with AZD0530 shows dose-dependent decrease in cell migration. (b) Paxillin, p130CAS and p-FAK (Y576/577) phosphorylation were inhibited in DU145 and PC3, following AZD0530 treatment for 30 min. Columns, mean; bars, standard error; \* $P < 0.05$  ( $n = 3$ ); \*\* $P < 0.01$  ( $n = 3$ ).



**Figure 5** AZD0530 inhibits tumor growth *in vivo*. (a) 25 mg/kg of AZD0530 was administered orally daily starting 2 days after orthotopic injection of 2 million DU145 cells. Mice were euthanized after 54 days. Established tumors were harvested and weighed. (b) Immunohistochemical analysis of tumor samples from (a) using specific phospho-Src Y419 antibody as described. Dots and triangles, tumor samples; columns, mean; bars, standard error; \* $P < 0.05$  ( $n = 10$ ).

#### *AZD0530 is a promising inhibitor of prostate cancer growth in orthotopic SCID mice model*

Utilizing the data we gathered regarding the effects of AZD0530 on *in vitro* growth and migration of prostate cancer cells, we tested its efficacy *in vivo* using orthotopically implanted DU145 in mice as our xenograft model. Xenograft mice receiving daily AZD0530 starting 2 days after the implantation have on average 45% smaller ( $P < 0.01$ ) tumor than control mice (Figure 5a). Src inhibition *in vivo* by AZD0530 was verified by immunohistochemistry (Figure 5b). Since AZD0530 treatment started shortly after implantation, the decreased tumor size xenograft mice treated with AZD0530 is consistent with the *in vitro* data of growth inhibition versus apoptosis. This is significant, as previous studies with other Src inhibitors revealed mostly inhibitory effects on metastasis rather than growth. We were unable to study the effect of AZD0530 on metastasis, as orthotopically implanted DU145 does not metastasize to any significant extent.

#### Discussion

Src is involved in prostate cancer growth and migration (Lee *et al.*, 2001, 2004; Nam *et al.*, 2005b; Kotha *et al.*, 2006). We previously reviewed Src's role (Chang *et al.*, 2007) in prostate cancer and wished to further characterize it. We therefore utilized AZD0530 to facilitate identification of Src-driven cell proliferation and migration signaling pathways in prostate cancer. Our data provide further understanding to foster correlative studies and translational research initiatives.

We found an association between higher relative Src activation and aggressive cell phenotypes. There are two Src isoforms and their expression levels are cell line-dependent. The origin of these isoforms is presently unclear. Also interesting is that cells with the lowest activated/total Src ratios (LAPC-4, PZ-HPV7, RWPE-1) also express the most Src. A possible explanation is that highly active Src is polyubiquitinated and thus quickly degraded (Hakak and Martin, 1999).

In our studies with AZD0530, we see a temporal sequence of its effects. As AZD0530 inhibits Src, changes to phosphorylation signals downstream occurred within minutes. The inhibited phosphorylation of FAK, p130CAS and paxillin quickly decreased cell migration. Taking into account the time it takes for transcriptional inhibition and protein degradation, changes in cyclin D1 and c-Myc levels are relatively late events seen hours post-treatment. Finally, consistent with cell doubling times, cell cycle changes and differential proliferation rates are observed days post-treatment.

We inhibited cell migration and proliferation using AZD0530. Although mechanistic studies of cell migration signaling did not reveal significant mechanistic differences between DU145 and PC3, they appear to regulate cell proliferation through c-Myc and cyclin D1 in different ways. Both cyclin D1 and c-Myc levels are more responsive to AZD0530 in DU145 than PC3. This is attributable to ERK1/2 being active and sensitive to AZD0530 in DU145 but not PC3. Since PC3 has no constitutively active ERK1/2, it alternatively regulates cyclin D1 and c-Myc through the Src-Ras-AKT-GSK3 $\beta$  pathway (Diehl *et al.*, 1998; Morin, 1999; Daaka, 2002). Interestingly,  $\beta$ -catenin but not GSK3 $\beta$  is affected in a dose-dependent manner by AZD0530 in DU145. Possible explanations of this finding include Src-mediated  $\beta$ -catenin synthesis (Karni *et al.*, 2005) and phosphorylation (Bjelfman *et al.*, 1990), thus resulting in increased stability (Roura *et al.*, 1999). Common to both cell lines, however, is that Src does not regulate cyclin D1 and c-Myc through STAT3.

Although our studies show that AZD0530 inhibits cell proliferation and migration through various signaling factors, they have relatively higher IC<sub>50</sub> values than Src autophosphorylation. Dose-dependent inhibition demonstrated in these assays suggests that Src contributes to their regulation. Nonspecific AZD0530 inhibition, however, cannot be excluded. Nevertheless, there are alternative explanations for these findings. Since phosphorylation status of proteins are dynamic systems dependent on the summative velocities of kinases and phosphatases, partial inhibition of kinase activity may

not be sufficient to allow phosphorylation status changes if phosphatase velocity remains less than kinase velocity. Furthermore, as shown through c-Myc in DU145 (Figure 3a), the accuracy of determining IC<sub>50</sub> values is dependent on the timing of the assay if Src is not the sole regulator of the factor in question, as 1  $\mu$ M of AZD0530 decreases c-Myc levels by 90% at 8 h but 50% at 24 h. Actual c-Myc AZD0530 IC<sub>50</sub> is therefore less than 1  $\mu$ M. Another explanation is that very little Src may be required to activate downstream signals. Individual kinases, such as ERK1/2, have been shown to display cooperative kinetics, which cumulatively in a signal-transduction chain is ultrasensitive to activation, akin to an on-off switch response (Li and Qian, 2003). In other words, very low initial activation of the upstream factor in a signal-transduction chain can be amplified and lead to changes downstream. Our kinetics study of c-Myc, which is regulated by ERK1/2, supports this hypothesis as very small amount of Src activation correlates with a relatively large rise in c-Myc levels (Figure 3a). The combination of ultrasensitivity and dose-dependent residual Src activity at  $\mu$ M AZD0530 concentrations (data not shown) suggests that the effect of Src inhibition on downstream factors decreases exponentially with increasing AZD0530, and therefore increases the IC<sub>50</sub> values of Src downstream factors. Extrapolating this further, we can see how the IC<sub>50</sub> values of transcription/translation and cell proliferation and migration involving a multitude of factors, many of which are not regulated by Src, can be significantly higher than Src.

The complexity of linking dose inhibition of Src phosphorylation with linear dose inhibition of other molecules and biological events is evident in the dosing and temporal data we present. Although AZD0530 may have other effects, we show it essential to the pathways and events presented. The decreased *in vivo* tumor growth correlates with significant inhibition of Src autophosphorylation by immunohistochemistry, demonstrating biological and translational relevance. AZD0530 represents an oral drug of low toxicity potentially of high value in the targeted therapy of prostate cancer. The mechanistic differences between the two androgen-independent prostate cancer cell lines DU145 and PC3 highlight the importance of an individualized, pharmacogenomic approach to patients. Studies such as ours are important in linking disease, detailed oncogenic pathway analysis and a targeted therapy *in vitro* and *in vivo*. These data have direct translational application to prostate cancer patients entering clinical trials using AZD0530.

## Materials and methods

### Cells and reagents

LNCaP, DU145, PC3, RWPE-1, PZ-HPV7 were obtained from American Type Culture Collection (Manassas, VA, USA). LAPC-4 was provided by Dr Sawyers (Department of Medicine, University of California at Los Angeles, Los Angeles, CA, USA). CWR22Rv1 was provided by Dr Pretlow

(Department of Pathology, Case Western Reserve University, Cleveland, OH, USA). Cell cultures were maintained in RPMI-1640 (Life Technologies Inc., Rockville, MD, USA) with 10% (LNCaP, RWPE-1), 5% (DU145, PC3, CWR22Rv1) fetal bovine serum, Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (LAPC-4) or keratinocyte serum-free medium with 5 ng/ml human recombinant epidermal growth factor and 50  $\mu$ g/ml bovine pituitary extract (PZ-HPV7) supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37 °C with 5% CO<sub>2</sub>. Polyclonal antibodies to AKT, p-AKT (S473), caspase 3, ERK1/2, p-FAK (Y576/577), p-GSK3 $\alpha/\beta$  (S21/9), p-p130CAS (Y410), paxillin, p-paxillin (Y118), p-Src (Y419) and STAT3 were obtained from Cell Signaling Technologies (Cambridge, MA, USA). Polyclonal antibodies to FAK and cyclin D1 were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Monoclonal antibodies to p-ERK1/2 (T202/Y204) and p-STAT3 (Y705) were obtained from Cell Signaling Technologies. Monoclonal antibodies to c-Myc and  $\alpha$ -tubulin were obtained from Santa Cruz Biotechnology. Monoclonal antibodies to p-FAK (Y397) and p130CAS were obtained from BD Biosciences (San Jose, CA, USA). Monoclonal antibodies to c-Myc and  $\beta$ -catenin were obtained from Santa Cruz Biotechnology. Monoclonal antibodies to GSK3 $\beta$ , Src and  $\beta$ -actin were obtained from Cell Signaling Technologies, Upstate Biotechnology and Sigma-Aldrich (St Louis, MO, USA), respectively. AZD0530 was obtained from AstraZeneca International (Alderley Park, UK). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide was obtained from Sigma-Aldrich. The DAKO Envision+ Kit was obtained from DAKO North American Inc. (Carpinteria, CA, USA). Diff-Quick set was purchased from Dade Behring Inc. (Newark, DE, USA). Dimethyl sulfoxide was obtained from Fisher Scientific (Pittsburgh, PA, USA). DNase-free RNase was obtained from Fermentas (Hanover, MD, USA). Fibronectin was obtained from Roche Applied Science (Indianapolis, IN, USA). Propidium iodide was obtained from Boehringer Mannheim Corporation (Indianapolis).

### Boyden chamber cell migration assay

Cell migration assay was performed as described previously and performed in triplicates (Evans *et al.*, 1991). Lower wells of the microchamber were filled with 50  $\mu$ g/ml of fibronectin in 0.1% BSA phenol-red free RPMI-1640 media as chemoattractant. Both chambers contained varying concentrations of AZD0530 (0–2  $\mu$ M). Cells were allowed to migrate for 4 h followed by Diff-Quick stain and counted as an average of five fields.

### Cell cycle analysis

Cells were plated in triplicate in 60 mm dishes followed by AZD0530 (1  $\mu$ M) treatment for 48 (DU145) and 72 (PC3) hours, accounting for slower proliferation rate in PC3 cells. Growth media were removed and saved. Cells were washed with phosphate-buffered saline (PBS) and the wash saved with the growth media. Remaining cells were trypsinized and placed together with growth media and PBS. Cells were pelleted and resuspended in 75% ethanol followed by overnight storage at –20 °C. Cells were centrifuged, washed with PBS, resuspended in PBS containing 10  $\mu$ g/ml DNase-free RNase, and incubated in 37 °C for 45 min. Final propidium iodide concentration of 0.05 mg/ml was added and incubated at room temperature for 20 min. Cell clumps were filtered. Cell DNA content was measured on Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA) and cell cycle phase was analysed

using Phoenix Multicycle (Phoenix Flow Systems, San Diego, CA, USA).

#### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed as described previously (Vinall *et al.*, 2006). Primers for cyclin D1 and c-Myc promoter regions are as follows: 5'-GCTC TCCACTTGCCCCTTTTA-3' (c-Myc, forward), 5'-GTTCCC AATTCTCAGCC-3' (c-Myc, reverse), 5'-GGGAGGAATT CACCCTGAAA-3' (cyclin D1, forward), 5'-CCTGCCCA AATTAAGAAA-3' (cyclin D1, reverse).

#### 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide cell proliferation assay

Cells were seeded overnight 2000 cells per well in triplicate in 96-well plates followed by single treatment of AZD0530 (62.5 nM–16 µM). On post-treatment days 1, 3 and 5, growth medium was removed followed by addition of 0.2 ml dimethyl sulfoxide per well and continuous shaking of plates at 200 rotations per minute for 15 min. Colorimetric measurement was performed at 450 nm.

#### Orthotopic mouse model

Severe combined immunodeficiency (CB17) mice 4 weeks of age were obtained from Harlan Sprague-Dawley and housed in pathogen-free conditions. Mice were placed in anesthesia with 2% isoflurane air. Two million DU145 cells were mixed with Matrigel in 1:1 ratio by volume and injected into a lateral lobe of the prostate as previously described (Stephenson *et al.*, 1992). Twenty-five milligrams per kilogram of AZD0530 dissolved in 0.5% hydroxypropyl methylcellulose (Sigma-Aldrich), 0.1% Tween 80 (Sigma-Aldrich) was orally given daily 2 days post-operation. Mice were euthanized 54 days post-operation and tumors harvested. Animal housing and experimental conditions were in compliance with the protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

## References

- Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A *et al.* (1995). Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J Biol Chem* **270**: 23589–23597.
- Bang YJ, Pirnia F, Fang WG, Kang WK, Sartor O, Whitesell L *et al.* (1994). Terminal neuroendocrine differentiation of human prostate carcinoma cells in response to increased intracellular cyclic AMP. *Proc Natl Acad Sci USA* **91**: 5330–5334.
- Barone MV, Courtneidge SA. (1995). Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. *Nature* **378**: 509–512.
- Biscardi JS, Ishizawa RC, Silva CM, Parsons SJ. (2000). Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res* **2**: 203–210.
- Bjelfman C, Meyerson G, Cartwright CA, Mellstrom K, Hammerling U, Pahlman S. (1990). Early activation of endogenous pp60src kinase activity during neuronal differentiation of cultured human neuroblastoma cells. *Mol Cell Biol* **10**: 361–370.
- Bowman T, Broome MA, Sinibaldi D, Wharton W, Pledger WJ, Sedivy JM *et al.* (2001). Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc Natl Acad Sci USA* **98**: 7319–7324.
- Bromann PA, Korkaya H, Courtneidge SA. (2004). The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23**: 7957–7968.

#### Reverse transcription-PCR

The Versagene RNA purification kit (Qiagen USA, Valencia, CA, USA) was used for mRNA extraction as per the manufacturer's instructions. RNA was reverse transcribed to cDNA using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) as per the manufacturer's instructions. Reverse transcriptase products were used as templates for PCR. The primers are as follows: 5'-ACCGAGGAGAATGTCAAGAGGC-3' (c-Myc, forward), 5'-CGTCGTTTCCGCAACAAGTC-3' (c-Myc, reverse), 5'-TGTTTGCAAGCAGGACTTTG-3' (cyclin D1, forward), 5'-TCATCCTGGCAATGTGAGAA-3' (cyclin D1, reverse).

#### Statistics

Data were analysed using Statview version 5.1 (SAS, Cary, NC, USA).

#### Western blotting

Western blotting was performed as described previously (Qiu *et al.*, 1998). Membranes were incubated overnight in 4 °C with primary antibodies in 5% non-fat milk tris-buffered saline Tween-20 followed by wash and 1-h room temperature incubation with respective horseradish peroxidase-conjugated secondary antibodies. Antibody-epitope binding was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

#### Acknowledgements

This study was supported by National Institutes of Health Grant KO8 DK60748-01 (CPE), RO1 DK52659 (H-JK) and Department of Defense Grant PC040161 (CPE). The contents of this report are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or the Department of Defense.

- Chang YM, Kung HJ, Evans CP. (2007). Nonreceptor tyrosine kinases in prostate cancer. *Neoplasia* **9**: 90–100.
- Chen G, Shukeir N, Potti A, Sircar K, Aprikian A, Goltzman D *et al.* (2004). Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. *Cancer* **101**: 1345–1356.
- Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. (2005). The Akt/PKB pathway: molecular target for cancer drug discovery. *Oncogene* **24**: 7482–7492.
- Cronauer MV, Schulz WA, Ackermann R, Burchardt M. (2005). Effects of WNT/beta-catenin pathway activation on signaling through T-cell factor and androgen receptor in prostate cancer cell lines. *Int J Oncol* **26**: 1033–1040.
- Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**: 785–789.
- Daaka Y. (2002). Mitogenic action of LPA in prostate. *Biochim Biophys Acta* **1582**: 265–269.
- de la Taille A, Rubin MA, Chen MW, Vacherot F, de Medina SG, Burchardt M *et al.* (2003). Beta-catenin-related anomalies in apoptosis-resistant and hormone-refractory prostate cancer cells. *Clin Cancer Res* **9**: 1801–1807.
- Desai SJ, Ma AH, Tepper CG, Chen HW, Kung HJ. (2006). Inappropriate activation of the androgen receptor by nonsteroids: involvement of the Src kinase pathway and its therapeutic implications. *Cancer Res* **66**: 10449–10459.

- Devi GR, Oldenkamp JR, London CA, Iversen PL. (2002). Inhibition of human chorionic gonadotropin beta-subunit modulates the mitogenic effect of c-myc in human prostate cancer cells. *Prostate* **53**: 200–210.
- Diehl JA, Cheng M, Roussel MF, Sherr CJ. (1998). Glycogen synthase kinase-3 $\beta$  regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* **12**: 3499–3511.
- Dominguez-Sola D, Dalla-Favera R. (2004). PINning down the c-Myc oncoprotein. *Nat Cell Biol* **6**: 288–289.
- Dong JT. (2006). Prevalent mutations in prostate cancer. *J Cell Biochem* **97**: 433–447.
- Evans CP, Walsh DS, Kohn EC. (1991). An autocrine motility factor secreted by the Dunning R-3327 rat prostatic adenocarcinoma cell subtype AT2.1. *Int J Cancer* **49**: 109–113.
- Farkas A, Szatmari E, Orbok A, Wilhelm I, Wejksza K, Nagyoszi P et al. (2005). Hyperosmotic mannitol induces Src kinase-dependent phosphorylation of beta-catenin in cerebral endothelial cells. *J Neurosci Res* **80**: 855–861.
- Furstoss O, Dorey K, Simon V, Barila D, Superti-Furga G, Roche S. (2002). c-Abl is an effector of Src for growth factor-induced c-myc expression and DNA synthesis. *EMBO J* **21**: 514–524.
- Gao L, Zhang L, Hu J, Li F, Shao Y, Zhao D et al. (2005). Down-regulation of signal transducer and activator of transcription 3 expression using vector-based small interfering RNAs suppresses growth of human prostate tumor *in vivo*. *Clin Cancer Res* **11**: 6333–6341.
- Golas JM, Arndt K, Etienne C, Lucas J, Nardin D, Gibbons J et al. (2003). SKI-606, a 4-anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. *Cancer Res* **63**: 375–381.
- Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS et al. (2005). HIF-1 $\alpha$ , STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. *Oncogene* **24**: 3110–3120.
- Guo Z, Dai B, Jiang T, Xu K, Xie Y, Kim O et al. (2006). Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell* **10**: 309–319.
- Hakak Y, Martin GS. (1999). Ubiquitin-dependent degradation of active Src. *Curr Biol* **9**: 1039–1042.
- Hauck CR, Hsia DA, Puente XS, Cheresh DA, Schlaepfer DD. (2002). FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth. *EMBO J* **21**: 6289–6302.
- Hennequin LF, Allen J, Breed J, Curwen J, Fennell M, Green TP et al. (2006). N-(5-Chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperazin-1-yl)ethoxy]-5-(tetrahydro-2H-pyran-4-yloxy)quinazolin-4-amine, a novel, highly selective, orally available, dual-specific c-Src/Abl kinase inhibitor. *J Med Chem* **49**: 6465–6488.
- Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI. (2006). Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* **97**: 263–274.
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T et al. (2008). Cancer statistics, 2008. *CA Cancer J Clin* **58**: 71–96.
- Karni R, Gus Y, Dor Y, Meyuhav O, Levitzki A. (2005). Active Src elevates the expression of beta-catenin by enhancement of cap-dependent translation. *Mol Cell Biol* **25**: 5031–5039.
- Kim D, Rath O, Kolch W, Cho KH. (2007). A hidden oncogenic positive feedback loop caused by crosstalk between Wnt and ERK pathways. *Oncogene* **26**: 4571–4579.
- Kotha A, Sekharam M, Cilenti L, Siddiquee K, Khaled A, Zervos AS et al. (2006). Resveratrol inhibits Src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein. *Mol Cancer Ther* **5**: 621–629.
- Lee D, Gautschi O. (2006). Clinical development of SRC tyrosine kinase inhibitors in lung cancer. *Clin Lung Cancer* **7**: 381–384.
- Lee LF, Guan J, Qiu Y, Kung HJ. (2001). Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. *Mol Cell Biol* **21**: 8385–8397.
- Lee LF, Louie MC, Desai SJ, Yang J, Chen HW, Evans CP et al. (2004). Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene* **23**: 2197–2205.
- Li G, Qian H. (2003). Sensitivity and specificity amplification in signal transduction. *Cell Biochem Biophys* **39**: 45–59.
- Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC, Behnia K et al. (2004). Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem* **47**: 6658–6661.
- Morin PJ. (1999). beta-catenin signaling and cancer. *Bioessays* **21**: 1021–1030.
- Nam S, Buettner R, Turkson J, Kim D, Cheng JQ, Muehlbauer S et al. (2005a). Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc Natl Acad Sci USA* **102**: 5998–6003.
- Nam S, Kim D, Cheng JQ, Zhang S, Lee JH, Buettner R et al. (2005b). Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res* **65**: 9185–9189.
- Park SI, Zhang J, Phillips KA, Araujo JC, Najjar AM, Volgin AY et al. (2008). Targeting SRC family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model. *Cancer Res* **68**: 3323–3333.
- Parsons JT. (2003). Focal adhesion kinase: the first ten years. *J Cell Sci* **116**: 1409–1416.
- Planas-Silva MD, Bruggeman RD, Grenko RT, Stanley Smith J. (2006). Role of c-Src and focal adhesion kinase in progression and metastasis of estrogen receptor-positive breast cancer. *Biochem Biophys Res Commun* **341**: 73–81.
- Prathapam T, Tegen S, Oskarsson T, Trump A, Martin GS. (2006). Activated Src abrogates the Myc requirement for the G0/G1 transition but not for the G1/S transition. *Proc Natl Acad Sci USA* **103**: 2695–2700.
- Qiu Y, Robinson D, Pretlow TG, Kung HJ. (1998). Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. *Proc Natl Acad Sci USA* **95**: 3644–3649.
- Quelle DE, Ashmun RA, Shurtleff SA, Kato JY, Bar-Sagi D, Roussel MF et al. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev* **7**: 1559–1571.
- Roura S, Miravet S, Piedra J, Garcia de Herreros A, Dunach M. (1999). Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. *J Biol Chem* **274**: 36734–36740.
- Rovin JD, Frierson Jr HF, Ledin W, Parsons JT, Adams RB. (2002). Expression of focal adhesion kinase in normal and pathologic human prostate tissues. *Prostate* **53**: 124–132.
- Schaller MD. (2001). Biochemical signals and biological responses elicited by the focal adhesion kinase. *Biochim Biophys Acta* **1540**: 1–21.
- Sears RC. (2004). The life cycle of C-myc: from synthesis to degradation. *Cell Cycle* **3**: 1133–1137.
- Slack JK, Adams RB, Rovin JD, Bissonette EA, Stoker CE, Parsons JT. (2001). Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. *Oncogene* **20**: 1152–1163.
- Steiner MS, Anthony CT, Lu Y, Holt JT. (1998). Antisense c-myc retroviral vector suppresses established human prostate cancer. *Hum Gene Ther* **9**: 747–755.
- Stephenson RA, Dinney CP, Gohji K, Ordonez NG, Killion JJ, Fidler IJ. (1992). Metastatic model for human prostate cancer using orthotopic implantation in nude mice. *J Natl Cancer Inst* **84**: 951–957.
- Taj MM, Tawil RJ, Engstrom LD, Zeng Z, Hwang C, Sanda MG et al. (2001). Mxi1, a Myc antagonist, suppresses proliferation of DU145 human prostate cells. *Prostate* **47**: 194–204.

- Tsai YT, Su YH, Fang SS, Huang TN, Qiu Y, Jou YS *et al.* (2000). Etk, a Btk family tyrosine kinase, mediates cellular transformation by linking Src to STAT3 activation. *Mol Cell Biol* **20**: 2043–2054.
- Unni E, Sun S, Nan B, McPhaul MJ, Cheskis B, Mancini MA *et al.* (2004). Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res* **64**: 7156–7168.
- Verras M, Sun Z. (2006). Roles and regulation of Wnt signaling and beta-catenin in prostate cancer. *Cancer Lett* **237**: 22–32.
- Vinall RL, Tepper CG, Shi XB, Xue LA, Gandour-Edwards R, de Vere White RW. (2006). The R273H p53 mutation can facilitate the androgen-independent growth of LNCaP by a mechanism that involves H2 relaxin and its cognate receptor LGR7. *Oncogene* **25**: 2082–2093.
- Watanabe G, Howe A, Lee RJ, Albanese C, Shu IW, Karnezis AN *et al.* (1996). Induction of cyclin D1 by simian virus 40 small tumor antigen. *Proc Natl Acad Sci USA* **93**: 12861–12866.
- Yeaman TJ. (2004). A renaissance for SRC. *Nat Rev Cancer* **4**: 470–480.
- Yuan ZL, Guan YJ, Chatterjee D, Chin YE. (2005). Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science* **307**: 269–273.
- Zhu S, Bjorge JD, Fujita DJ. (2007). PTP1B contributes to the oncogenic properties of colon cancer cells through Src activation. *Cancer Res* **67**: 10129–10137.

# Regulation of Id1 Expression by Src: Implications for Targeting of the Bone Morphogenetic Protein Pathway in Cancer

Oliver Gautschi,<sup>1,2</sup> Clifford G. Tepper,<sup>2,3</sup> Phillip R. Purnell,<sup>2</sup> Yoshihiro Izumiya,<sup>3</sup> Christopher P. Evans,<sup>4</sup> Tim P. Green,<sup>5</sup> Pierre Y. Desprez,<sup>6</sup> Primo N. Lara,<sup>2</sup> David R. Gandara,<sup>2</sup> Philip C. Mack,<sup>2</sup> and Hsing-Jien Kung<sup>2,3</sup>

<sup>1</sup>Department of Medical Oncology, Bern University Hospital, Bern, Switzerland; <sup>2</sup>University of California Davis Cancer Center, Departments of <sup>3</sup>Biochemistry and Molecular Medicine, and <sup>4</sup>Urology, School of Medicine, University of California Davis, Sacramento, California; <sup>5</sup>AstraZeneca Pharmaceuticals, Alderley Park, United Kingdom; and <sup>6</sup>California Pacific Medical Center, Cancer Research Institute, San Francisco, California

## Abstract

**Deregulated activation of the Src tyrosine kinase and heightened Id1 expression are independent mediators of aggressive tumor biology. The present report implicates Src signaling as a critical regulator of Id1 gene expression. Microarray analyses showed that Id family genes were among the most highly down-regulated by incubation of A549 lung carcinoma cells with the small-molecule Src inhibitor AZD0530. Id1 transcript and protein levels were potently reduced in a dose-dependent manner concomitantly with the reduction of activated Src levels. These effects were conserved across a panel of lung, breast, prostate, and colon cancer cell lines and confirmed by the ability of PP2, Src siRNA, and Src-blocking peptides to suppress Id1 expression. PP2, AZD0530, and dominant-negative Src abrogated Id1 promoter activity, which was induced by constitutively active Src. The Src-responsive region of the Id1 promoter was mapped to a region 1,199 to 1,360 bps upstream of the translation start site and contained a Smad-binding element. Src was also required for bone morphogenetic protein-2 (BMP-2)-induced Id1 expression and promoter activity, was moderately activated by BMP-2, and complexed with Smad1/5. Conversely, Src inhibitors blocked Smad1/5 nuclear translocation and binding to the Src-responsive region of the Id1 promoter. Consistent with a role for Src and Id1 in cancer cell invasion, Src inhibitors and Id1 siRNA decreased cancer cell invasion, which was increased by Id1 overexpression. Taken together, these results reveal that Src positively interacts with the BMP-Smad-Id pathway and provide new ways for targeted inhibition of Id1. [Cancer Res 2008;68(7):2250–8]**

## Introduction

The Src family of nonreceptor protein tyrosine kinases contains nine members, including Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk, and Yrk (1). Src is activated by growth factor receptors, cytokine receptors, protein tyrosine phosphatase 1B, CAS, and focal adhesion kinase (FAK). Src interacts with a network of intracellular signaling pathways, including the integrin/FAK pathway,  $\beta$ -catenin/Wnt, RAS-MEK, phosphatidylinositol-3-OH kinase-AKT

and Janus-activated kinase-STAT pathways. These complex interactions explain why Src is involved in a large number of cellular functions including adhesion, migration, invasion, survival, proliferation, differentiation, inflammation, and angiogenesis. Activated Src induced transformation in fibroblasts, and Src kinases were found frequently to be overexpressed and activated in human cancer (2). This prompted the development of a number of small-molecule Src kinase inhibitors that reduced cancer invasion and metastasis in preclinical models. For example, AZD0530, a potent and selective small-molecule inhibitor of Src kinase, is currently being tested in phase II clinical trials in patients with cancer (3, 4). Apart from their promising clinical utility, small-molecule inhibitors of Src possess the potential to identify genes regulated by Src signaling and putative effector molecules.

The inhibitor of DNA binding/differentiation (Id) family of helix-loop-helix (HLH) proteins comprises four members (Id1–4) that all lack a DNA-binding domain (5). Id proteins associate with and inhibit the function of basic HLH transcription factors, including MyoD and E-proteins, to regulate normal cell fate determination, differentiation, and angiogenesis (6–9). Expression of Id1 is induced by bone morphogenetic proteins (BMP), which activate Smad1/5 via the BMP-receptors (10–13). Activated Smad1/5 binds Smad4, translocates to the nucleus, binds to Smad-binding elements in the Id1 promoter, and recruits transcription factors and coactivators that induce Id1 transcription (14–16). In contrast, transforming growth factor (TGF) $\beta$  can repress the Id1 promoter by activation of Smad3 (17). BMPs and Id1 are overexpressed in various cancer types and are associated with an aggressive, invasive phenotype (18–22). Expression of Id gene family expression is mediated by oncogenic RAS, MYC, and TP53 gain of function mutation (12, 23–25). Id1 promotes invasion by production of a 120-kDa gelatinase, mediates tumor angiogenesis by production of vascular endothelial growth factor, facilitates hormone-independent growth, and is involved in the resistance of cancer cells against cytotoxic drugs (19, 26, 27). Due to its role in cell differentiation and in vascular endothelial cells, Id1 has also been implicated in the biology of cancer stem cells and tumor angiogenesis (8, 28, 29). Altogether, there is strong evidence that Id1 is an interesting drug target in cancer (30). However, strategies of Id targeting have thus far been limited to methods of gene silencing in the laboratory. Thus, the availability of pharmaceutical methods to inhibit Id1 *in vivo* may greatly advance the understanding of the role of Id1 in the biology, therapy, and prevention of cancer.

Here, we show that Src interacts with and is a positive modulator of the BMP-2/Smad1/Id1 signaling pathway in lung cancer cells, suggesting an important role for Id1 in Src-mediated invasion. We also provide evidence that Src inhibition by small

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Requests for reprints:** Oliver Gautschi, Department of Medical Oncology, Bern University Hospital, 3010 Bern, Switzerland. Phone: 41-31-632-41-14; Fax: 41-31-632-41-20; E-mail: [oliver.gautschi@insel.ch](mailto:oliver.gautschi@insel.ch).

©2008 American Association for Cancer Research.  
doi:10.1158/0008-5472.CAN-07-6403

molecules significantly reduces the level of Id1 in lung, breast, prostate, and colon cancer cells, thereby providing a potential way to target Id1 *in vivo*.

## Materials and Methods

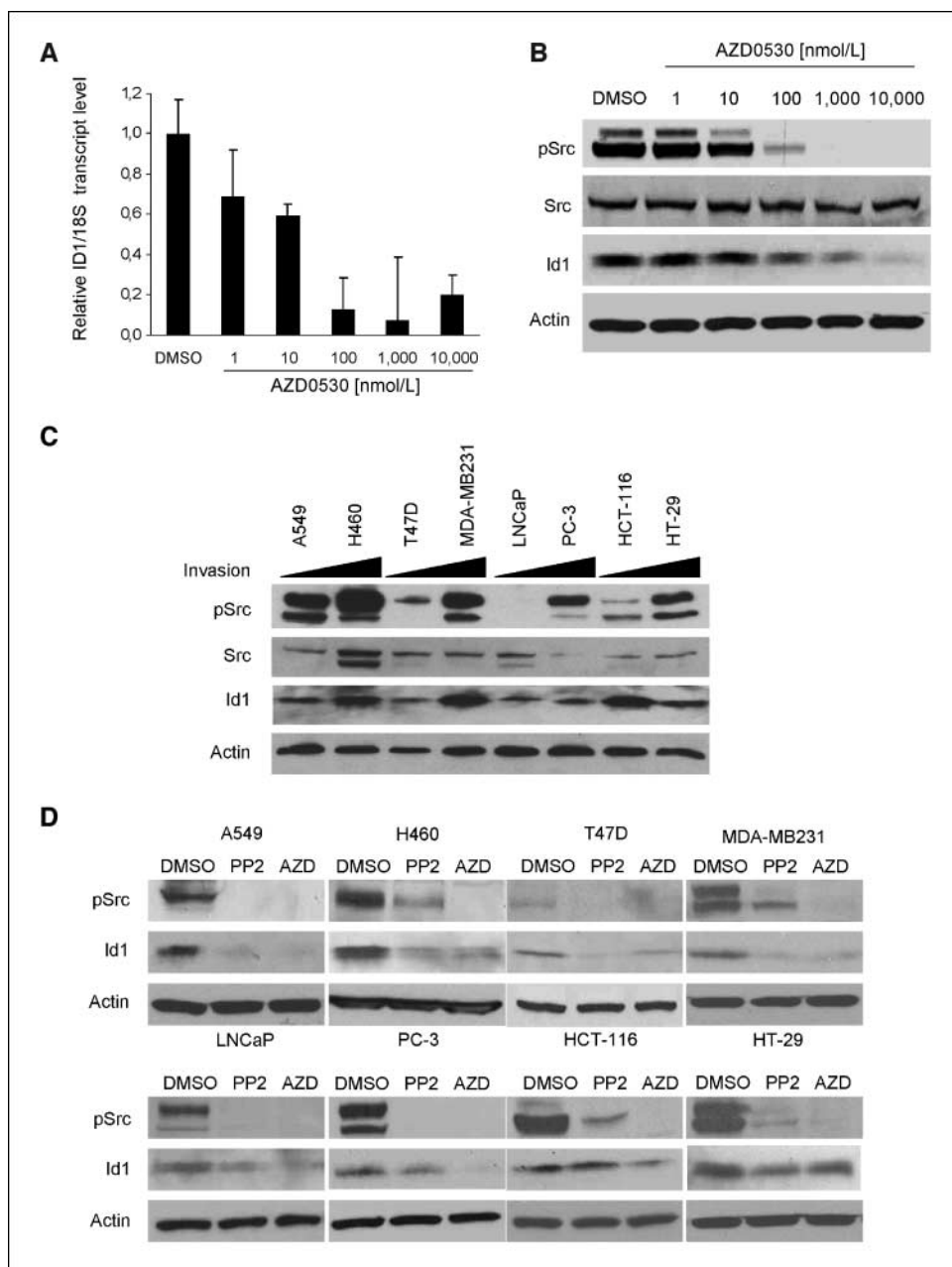
**Cell lines and reagents.** The following cell lines were obtained from the American Type Culture Collection: A549, H460, LNCaP, PC-3, T47D, MDA-MB231, HCT-116, and HT-29. Cells were cultured in RPMI (LNCaP and PC-3) or DMEM (all other cell lines) plus 10% filtered, heat-inactivated fetal bovine serum (FBS). PP2 (Calbiochem) and AZD0530 (AstraZeneca) were solubilized in DMSO to obtain a 10 and 1 mmol/L stock solution, respectively. The Src-blocking peptides CprYKYY- $\beta$ Ala-r7 and CprYKYY- $\beta$ Ala-k7 (provided by Dr. Kit Lam, University of California Davis Cancer Center, Sacramento, CA) were solubilized in sterile H<sub>2</sub>O to obtain a 25 mmol/L stock solution (31). Recombinant human BMP-2 (R&D Systems) was reconstituted in 4 nmol/L HCl containing 0.1% bovine serum albumin (BSA)

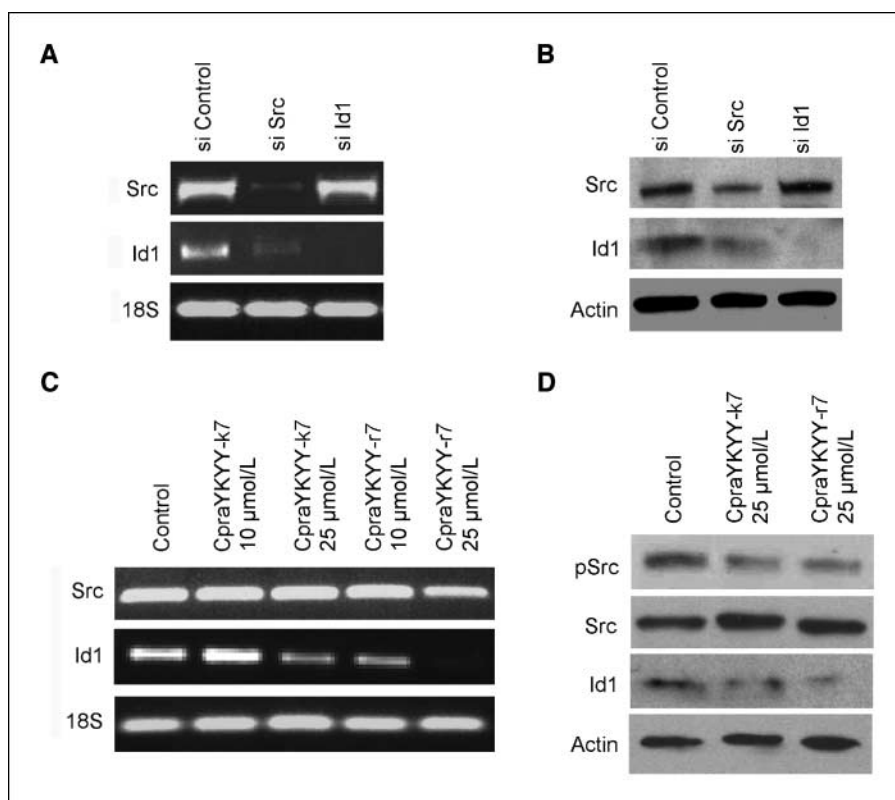
to obtain a 10  $\mu$ g/mL stock solution. Stock solutions were stored at  $-20^{\circ}\text{C}$  and diluted in DMEM for each experiment.

**Microarray gene expression profiling.** RNA isolation, RNA purification, and genome-wide expression profiling using Human Genome U133 Plus 2.0 GeneChip arrays (Affymetrix) was performed according to the manufacturer's protocols and as described previously (24). Initial data processing (e.g., signal detection and scaling) for each chip was performed using Affymetrix GeneChip Operating Software. Model-based expression analysis (using the perfect match-mismatch model) was used to identify differentially expressed genes using DNA-Chip Analyzer software (dChip; ref. 32). For this, signals from all of the arrays were normalized to the array that had the median overall intensity. Criteria for the selection of genes exhibiting significant expression changes included an average fold change of  $\geq 2.0$  (AZD0530/DMSO), *P* values of  $\leq 0.05$ , and at least 100 units of change between the two treatments being tested.

**Reverse transcription-PCR.** Total RNA was extracted using the Trizol protocol (Invitrogen) and cDNA was generated using M-MuLV reverse

**Figure 1.** Id gene expression is down-regulated by Src inhibition in cancer cells. A549 cells were incubated with increasing concentrations of AZD0530, and control cells were incubated with DMSO. *A*, RNA was isolated after 24 h, and real-time RT-PCR for Id1 RNA and 18S rRNA was performed in triplicates using Sybr Green. Id1 levels were normalized for 18S rRNA. *Columns*, mean relative to control; *bars*, SEs. *B*, protein was isolated after 48 h of incubation with AZD0530, and Western blotting was performed to determine the levels of pY419-Src, Src, Id1, and actin. *C*, lung (A549 and H460), breast (T47D and MDA-MB231), prostate (LNCaP and PC-3), and colon (HCT-116 and HT-29) cancer cells were selected according to their reported invasive potential, and Western blotting was performed to compare the basal levels of pY419-Src, Src, Id1, and actin. *D*, cells were incubated for 48 h with PP2 (10  $\mu$ mol/L) or AZD0530 (1  $\mu$ mol/L; AZD). Control cells were incubated with DMSO. Western blotting was performed to determine the levels of pY419-Src, Id1, and actin.





**Figure 2.** Reduction of Id1 mRNA and protein levels by Src siRNA and blocking peptides. **A**, A549 cells were incubated with Src siRNA, Id1 siRNA, or nontargeting control siRNA (each at 100 nmol/L; 24 h). RNA was isolated and RT-PCR was performed using specific primers for Src mRNA, Id1 mRNA, and 18S rRNA. **B**, protein was isolated for Western blotting and probing for Src, Id1, and actin. **C**, A549 cells were incubated with the Src-blocking peptides CpraYKYY- $\beta$ Ala-k7 and CpraYKYY- $\beta$ Ala-r7 at the concentrations indicated for 24 h. RNA was isolated and RT-PCR was performed using specific primers for Src mRNA, Id1 mRNA, and 18S rRNA. **D**, Western blotting was performed to determine the levels of pY419-Src, Src, Id1, and actin.

transcriptase (Fermentas). For PCR primer sequences and annealing temperatures, see Supplementary Fig. S2. PCR products were loaded onto 1% agarose gels and run at 110 V for 1 h.

**Real-time RT-PCR.** Quantitative real-time PCR was performed using iQ Sybr Green Supermix and the iCycler detection system (Bio-Rad). Primer sequences were as follows: ID1 forward, 5'-CTCTACGACATGAACGGCTGT-3'; ID1 reverse, 5'-TGCTCACCTTGCGGTTCTG-3'; 18S forward, 5'-CGCCGCT-AGAGGTGAAATTCT-3'; and 18S reverse, 5'-CGAACCTCCGACTTTCGTCT-3'. Standard dilutions, melting curve analysis, and agarose gel electrophoresis of PCR products were performed to confirm accuracy. Triplicate Id1 expression values were normalized for 18S rRNA, and data were processed using Q-GENE software (33).

**Western blotting.** Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer on ice for 30 min and protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Biotechnology). Equal amounts of protein were electrophoresed on polyacrylamide gradient gels (4–20%) and transferred onto nitrocellulose membranes by semidry blotting. Membranes were blocked for 1 h with TBS containing 5% nonfat dry milk and incubated overnight with antibodies against Src (Upstate), phospho-Src family (Cell Signaling), Id1 (Biocheck), pS463/465-Smad1/5 (Upstate), Smad1/5/8 (Santa Cruz Biotechnology), and  $\beta$ -Actin (Santa Cruz Biotechnology). Membranes were washed in TBS and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Promega). Visualization was performed using enhanced chemiluminescent (ECL) detection reagent and ECL X-ray films (Amersham).

**Immunoprecipitation.** Cells were lysed with RIPA buffer as described above. Equal amounts of protein were processed using the ExactCruz kit (Santa Cruz Biotechnology) following the manufacturer's protocol. Briefly, anti-Src antibody (Upstate) was mixed with immunoprecipitation matrix and incubated overnight at 4°C. The mix was centrifuged, and cell lysates were added and incubated overnight at 4°C on a rotator. After centrifugation, pellets were washed in PBS, resuspended in Laemmli buffer, and boiled at 95°C for 5 min. Samples were subjected to SDS-PAGE and Western blotting for Smad1/5/8 (Santa Cruz Biotechnology) and Src (Upstate).

**RNA interference.** Standard siCONTROL (D-001210-02), on-target plus SMART pool human Src (L-003175-00), and on-target plus SMART pool human Id1 (L-005051-00) were purchased (Dharmacon). Oligonucleotides were complexed with Lipofectamine in Opti-MEM (Invitrogen) according to the manufacturer's protocol and delivered to cells at a final concentration of 100 nmol/L.

**Id1 promoter assays.** A549 cells were transfected in 96-well plates for 24 h with previously described ID1pGL-luc reporter and SV40pRL coreporter plasmids at a ratio of 10:1 in the presence of Effectene (Qiagen) in 10% FBS DMEM (34). For Src inhibitor assays, cells were then incubated for 24 h with DMSO, PP2, or AZD0530 in 10% FBS DMEM. Samples were lysed and analyzed using the Dual-Luciferase Reporter Assay system (Promega) on a MicroLumat luminometer (EG & G Berthold). For Src mutant assays, cells were triple transfected for 24 h with ID1pGL, SV40pRL plus PCI vectors containing wild-type human Src, dominant-negative human SrcK298M (provided by Dr. Don Fujita, University of Calgary, Alberta, Canada), or constitutively active chicken SrcY527F (provided by Dr. June Zhou, University of California Davis Cancer Center, Sacramento, CA; ref. 35). Cells were then incubated in fresh 10% FBS DMEM for 18 h followed by serum starvation in DMEM for 6 h. For promoter region assays, cells were triple-transfected for 24 h with Src-Y527F, SV40pRL, plus full-length ID1pGL or one of seven previously described ID1 promoter 5' deletion constructs (34). Cells were then incubated in fresh 10% FBS DMEM for 18 h followed by serum starvation in DMEM for 6 h. Assays were performed in triplicates, firefly luciferase activity was normalized for *Renilla* luciferase activity, and relative Id1 promoter activity was calculated based on the mean value of the respective control.

**Chromatin immunoprecipitation.** A549 cells were incubated with DMSO, PP2, or AZD0530 for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). Cells were cross-linked with 1% formaldehyde for 10 min and incubated in 0.125 mol/L glycine for 5 min. Plates were scraped and cells were centrifuged. Pellets were resuspended in swelling buffer containing 100 mmol/L Tris, 10 mmol/L KOAc, 15 mmol/L MgOAc, and protease-inhibitor cocktail (Roche); incubated for 20 min on ice; and dounce homogenized 15 times. Nuclei were centrifuged; resuspended in

buffer containing 10 mmol/L EDTA, 50 mmol/L Tris-HCl, 0.5% SDS, and protease inhibitor cocktail; and sonicated using a BioRuptor (Diagenode). Lysates were diluted in buffer containing 150 mmol/L NaCl, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, 1% Triton-X, and protease inhibitor cocktail, and split into two aliquots for overnight incubation with 5  $\mu$ g of anti-Smad1/5/8 antibody (Santa Cruz Biotechnology) or rabbit IgG (Oncogene Science). Samples were then incubated with 5  $\mu$ g of sonicated salmon sperm DNA (Sigma-Aldrich) and 50  $\mu$ L of protein G agarose (Upstate) for 2 h. After centrifugation, supernatant was stored (input control) and pellets were washed in TSE buffer [1% TritonX-100, 0.1% SDS, 2 mmol/L EDTA, and 20 mmol/L Tris-HCl (pH 8.1)], eluted in TE buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA), and incubated at 65°C overnight to reverse the cross-linking. Samples were incubated with 1  $\mu$ L of Proteinase K (Fermentas) for 1 h at 55°C, and DNA was isolated using the QIAquick PCR purification kit (Qiagen). PCR for the Src-responsive region was performed using forward primer 5'-AATTGTTGGGATTACAGCGTG-3' and reverse primer 5'-CTGGGAATGCGTTTCTTGCG-3' at an annealing temperature of 55°C for 35 cycles. PCR products were separated on 1.5% agarose gels.

**Immunofluorescence.** A549 cells in chamber slides were incubated with DMSO, PP2, or AZD0530 for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). Cells were then fixed with 3.7% formaldehyde, permeabilized with Triton-100, blocked with 0.5% BSA in PBS, and incubated with anti-Smad1/5/8 antibody (Santa Cruz Biotechnology) overnight. Cells were washed and incubated with Alexa-647-conjugated

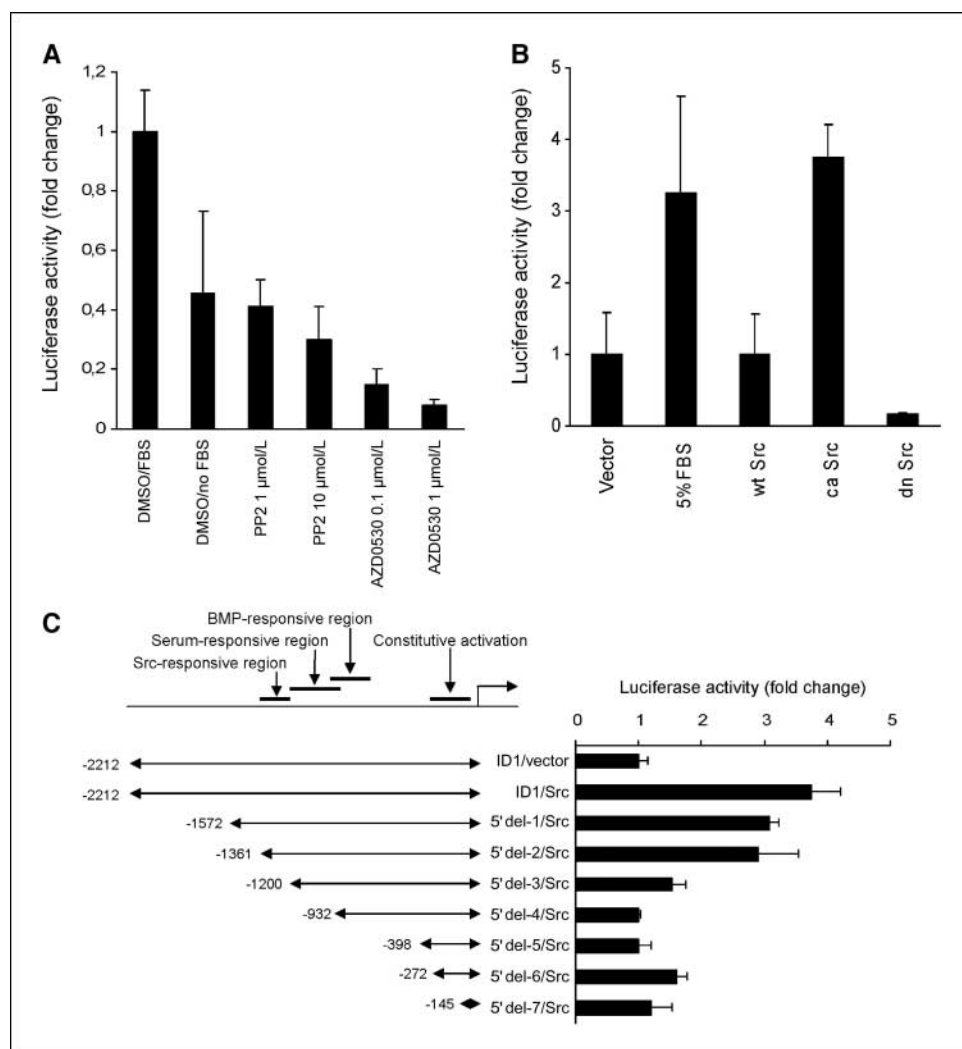
secondary antibody and Hoechst dye. After washing, slides were analyzed using a BX61 microscope and SlideBook 4.1 imaging software (Olympus).

**Establishment of a stable Id1-overexpressing A549 subline.** The full-length Id1 cDNA sequence was subcloned from pBabe-Id1 (26) into pLNCX2 retroviral vector (BD Clontech) for cytomegalovirus promoter-driven expression. For production of retrovirus, pLNCX2-Id1 and pLNCX2-empty expression constructs (2  $\mu$ g) were transfected into LinX-A amphotropic packaging cells using FuGENE 6 transfection reagent. Cultures were incubated at 32°C. After 72 h, virus-containing supernatant was collected, centrifuged at 3,000  $\times$  g for 15 min at 4°C, and filtered through a 0.45- $\mu$ m surfactant-free cellulose acetate membrane (Corning, Inc.). A549 cells were then infected with a mix of DMEM, virus-containing supernatant (1:1), and polybrene (4  $\mu$ g/mL). After incubation for 24 h at 32°C, cells were selected in 10% FBS DMEM with 400  $\mu$ g/mL of Geneticin (JR Scientific) at 37°C for 3 wk.

**Invasion assays.** Cell culture inserts with polyethylene terephthalate membranes and 8- $\mu$ m pores were coated with 60  $\mu$ L Matrigel (BD Biosciences). Top chambers were filled with A549 cells in 5% FBS DMEM containing siRNA, DMSO, PP2, or AZD0530. Bottom chambers were filled with 10% FBS DMEM. After 24 h, cells in the top chamber were removed with cotton swabs, and cells on the bottom side of the insert were fixed with 3.7% formaldehyde, stained with 0.5% methylene blue, and counted on an IX50 microscope (Olympus).

**Statistical analysis.** All experiments were performed at least thrice; values represent the mean of triplicate samples and SEs of the mean.

**Figure 3.** Identification of an Src-responsive region in the Id1 promoter. **A**, A549 cells were transfected for 24 h with ID1pGL firefly luciferase reporter plus SV40pRL *Renilla* luciferase coreporter followed by incubation for 24 h with DMSO, PP2, or AZD0530 at the concentrations indicated. Triplicate samples were measured using the dual luciferase reporter assay. **B**, A549 cells were triple-transfected with ID1pGL reporter, SV40pRL coreporter plus wild-type (wt) Src, constitutively active (ca) Src, or dominant-negative (dn) Src. Cells were then incubated in 10% FBS DMEM for 18 h followed by serum starvation for 6 h. **C**, A549 cells were triple-transfected for 24 h with constitutively active Src, and SV40pRL coreporter plus either the ID1 full-length promoter reporter construct or one of the Id1 promoter deletion reporter constructs (5' del-1 to 5' del-7). Cells were then incubated in 10% FBS DMEM for 18 h followed by serum starvation for 6 h.



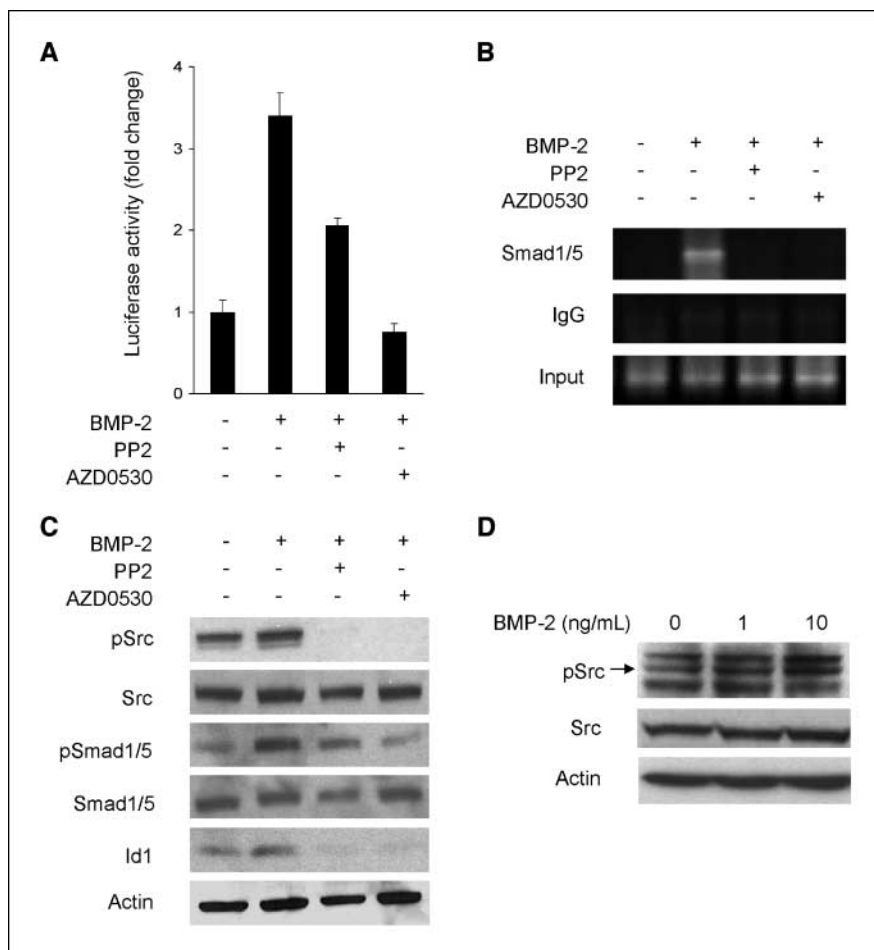
## Results

**Id gene expression is down-regulated by Src inhibition in cancer cells.** To identify novel downstream effectors of Src signaling in cancer, we performed genome-wide expression profiling of cells subjected to Src kinase inhibition. To this end, A549 lung adenocarcinoma cells were incubated for 24 hours with AZD0530 (750 nmol/L) in DMSO or with DMSO alone followed by RNA extraction and expression analysis using Affymetrix U133 plus 2.0 arrays. A total of 175 genes were differentially regulated ( $\geq 2$ -fold) in response to incubation with AZD0530. The genes most dramatically down-regulated by AZD0530 were the inhibitors of differentiation gene family members (ID1-4), inhibitory Smads (SMAD6 and SMAD7), TGF $\beta$ 1, and SERPINE1/PAI-1 (Supplementary Fig. S1). These findings were confirmed by standard reverse transcription PCR (RT-PCR), and the specificity of the effect of AZD0530 was shown by the fact that levels of Src and of 18S rRNA transcripts were unchanged (Supplementary Fig. S2). Taken together, these expression changes suggested that AZD0530-mediated Src inhibition leads to suppression of the Smad-Id signaling pathway.

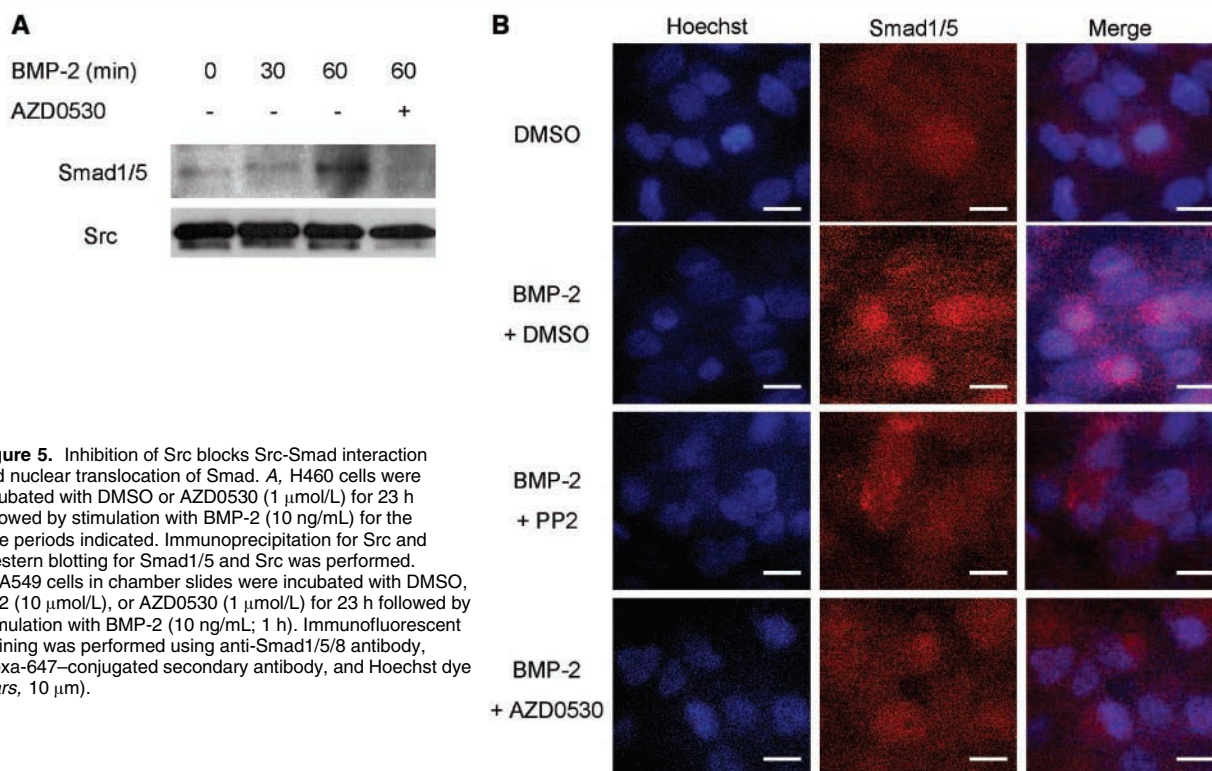
Based on its (a) strong association with cancer progression and (b) well-defined regulation by Smad signaling, we chose to focus on better defining the mechanism responsible for Id1 as a target of Src inhibition. To characterize further the effect of AZD0530 on Id1 mRNA levels, A549 cells were incubated with increasing concentrations of AZD0530 (1 nmol/L–10  $\mu$ mol/L) for 24 hours.

Quantitative real-time RT-PCR showed a dose-dependent reduction of Id1 expression by AZD0530, which reached a nadir at 100 nmol/L (Fig. 1A). Western blot analysis of companion cultures treated with AZD0530 for 48 hours was performed to show a correlation between AZD0530-mediated Src inhibition and down-regulation of Id1. The results confirmed a dose-dependent reduction in Id1 expression that corresponded with decreased levels of activated pY419-Src, whereas the levels of total Src were unchanged (Fig. 1B). The anti-phospho-Src family antibody detected multiple bands between 55 and 70 kDa, consistent with the presence of multiple Src family members in A549 cells. Reprobing with Src-specific antibody confirmed that the 60-kDa band represented the phosphorylated pp60<sup>c-Src</sup> protein.

To investigate the functional relationship between Src and Id1 and its therapeutic implications, we examined a panel of human cancer cell lines from four types of cancer, including lung (A549 and H460), breast (T47D and MDA-MB231), prostate (LNCaP and PC-3), and colon (HCT-116 and HT-29), each represented by a pair of cell lines with different invasive potential (36–39). Protein extracts of cells grown under normal conditions were subjected to Western blotting for pY419-Src, total Src, Id1, and actin (Fig. 1C). Immunoblot analysis showed that basal Src activity (pY419-Src) was easily detectable in six of eight cell lines. The levels of pY419-Src corresponded with the reported invasive potential in each pair. Similarly, Id1 levels corresponded with the reported invasive potential and level of pY419-Src level in six of eight cell lines.



**Figure 4.** Cooperation of Src with the BMP/Smad signaling pathway. **A**, A549 cells were transfected for 24 h with ID1pGL reporter and SV40pRL coreporter followed by incubation with DMSO, PP2 (10  $\mu$ mol/L), or AZD0530 (1  $\mu$ mol/L) for 23 h and then stimulated with BMP-2 (10 ng/mL; 1 h). Triplicate samples were analyzed using the dual luciferase reporter system. **B**, A549 cells were incubated with DMSO, PP2 (10  $\mu$ mol/L), or AZD0530 (1  $\mu$ mol/L) for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). ChIP was performed using anti-Smad1/5 antibody or normal rabbit IgG. DNA was extracted and the proposed Src-responsive region of the Id1 promoter was amplified by PCR. **C**, A549 cells were incubated for 23 h with DMSO, PP2 (10  $\mu$ mol/L), or AZD0530 (1  $\mu$ mol/L) followed by stimulation with BMP-2 (10 ng/mL; 1 h). Western blotting was performed to determine the levels of pY419-Src, Src, phospho-Smad1/5, Smad1/5, Id1, and actin. **D**, A549 cells were incubated for 1 h with BMP-2 at the concentrations indicated. Western blotting was performed to determine the levels of pY419-Src, Src, and actin.



**Figure 5.** Inhibition of Src blocks Src-Smad interaction and nuclear translocation of Smad. **A**, H460 cells were incubated with DMSO or AZD0530 (1  $\mu$ mol/L) for 23 h followed by stimulation with BMP-2 (10 ng/mL) for the time periods indicated. Immunoprecipitation for Src and Western blotting for Smad1/5 and Src was performed. **B**, A549 cells in chamber slides were incubated with DMSO, PP2 (10  $\mu$ mol/L), or AZD0530 (1  $\mu$ mol/L) for 23 h followed by stimulation with BMP-2 (10 ng/mL; 1 h). Immunofluorescent staining was performed using anti-Smad1/5/8 antibody, Alexa-647-conjugated secondary antibody, and Hoechst dye (bars, 10  $\mu$ m).

Although Id1 was highly expressed in the colon cancer cells HCT-116 and HT-29, an inverse relationship between pY419-Src and Id1 levels was observed. This suggests that Src is a major regulator of Id1 but that other pathways exist to activate Id1. Next, the effect of Src inhibition on Id1 expression in each cell line was determined (Fig. 1D). Incubation of the cells with PP2 (10  $\mu$ mol/L) or AZD0530 (1  $\mu$ mol/L) for 48 hours reduced the levels of pY419-Src and Id1 in all cell lines. These results showed an association between Src and Id1 expression, and that Src inhibitors can effectively mediate Id1 down-regulation.

**Reduction of Id1 mRNA and protein levels by Src siRNA and blocking peptides.** In addition to small-molecule inhibitors, two alternative approaches to Src-targeting were used to confirm the role of Src in the regulation of Id1 expression. In the first approach, A549 cells were transfected with an Src-specific siRNA pool, as well as an Id1-specific siRNA and a nontargeting control siRNA. After 24 hours, RNA and protein was isolated and RT-PCR and Western blotting was performed, respectively (Fig. 2A and B). The expected effect of each siRNA on its respective target was confirmed. Moreover, Src siRNA also reduced Id1 mRNA and protein levels. The levels of 18S rRNA and actin protein remained unchanged. Src siRNA had no visual effect on other members of the Src family tested (Supplementary Fig. S3). In the second approach, A549 cells were incubated for 24 hours with cell-permeable Src-blocking peptides CpraYKYY- $\beta$ Ala-k7 and CpraYKYY- $\beta$ Ala-r7 at the concentrations indicated (Fig. 2C and D; ref. 31). Target specificity of these peptides has been shown earlier (31). Both peptides reduced Id1 transcript levels in a dose-dependent manner. Western blotting showed a reduction in pY419-Src levels and Id1 protein levels, whereas total Src levels remained unchanged. These results

confirmed that inhibition of Src activity or expression leads to reduced Id1 levels.

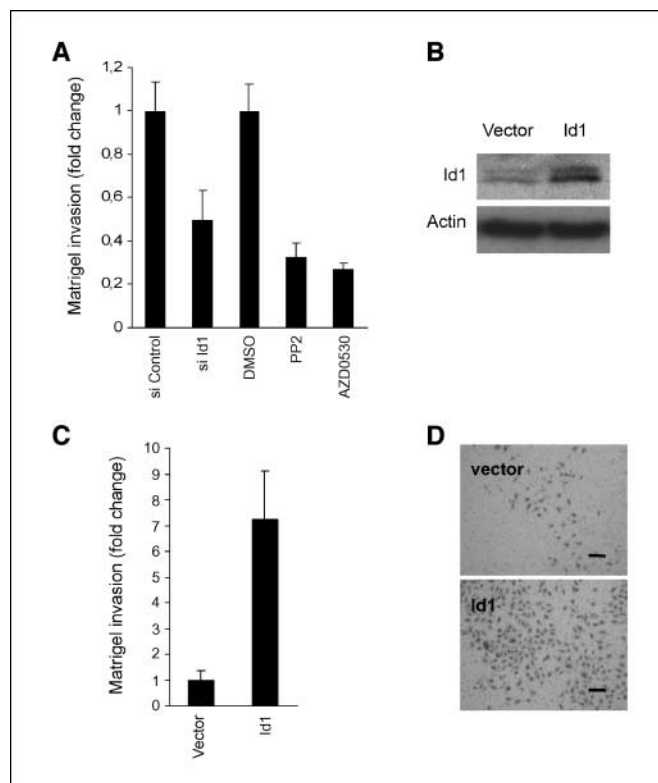
**Identification of an Src-responsive region in the human Id1 promoter.** To define further the role of Src in the regulation of Id1 expression, we used dual-luciferase reporter assays to monitor the activity of the Id1 promoter. A549 cells were cotransfected with ID1pGL firefly luciferase reporter (34) and SV40pRL *Renilla* luciferase coreporter for 24 hours followed by incubation with DMSO, PP2, or AZD0530 for an additional 24 hours. As a control, serum starvation for 24 hours decreased the Id1 promoter signal by 54% (Fig. 3A). In comparison, both PP2 and AZD0530 significantly reduced the Id1 promoter signal in a dose-dependent manner. Of note, AZD0530 (1  $\mu$ mol/L) in serum-stimulated A549 cells reduced Id1 promoter activity by >90%, suggesting that the promoter is strongly Src dependent. To investigate this further, we tested if Id1 promoter activity could be modulated by enforced expression of wild-type or mutant forms of Src. Under conditions of serum-deprivation, a constitutively active Src mutant resulted in a 3.7-fold induction in Id1 promoter activity, compared with a 3.2-fold induction by serum (Fig. 3B). In contrast, wild-type Src did not significantly alter Id1 promoter activity, whereas dominant-negative Src decreased endogenous (serum deprived) Id1 promoter activity by 85%. In an effort to map the region in the Id1 promoter that was responsible for Src-mediated activation, each of a set of 5'-deletion constructs (5'*del*-1 to 5'*del*-7) generated from the full-length (2.2 kbp) Id1 promoter and cloned into the pGL-luciferase reporter (34) were cotransfected with SV40pRL coreporter plus constitutively active Src (Fig. 3C). Again under conditions of serum deprivation, the greatest decrease in the Id1 promoter signal occurred with construct 5'*del*-3 (60% of the signal of the full-length

promoter construct), corresponding to a region between positions 1,360 and 1,199 in the Id1 promoter. Analysis of the sequence of this newly characterized Src-responsive region revealed the CAGC motif (positions 1,352–1,349), representing a putative Smad-binding element.

**Cross-talk between Src and BMP-Smad signaling pathway.** BMP-2 is a well-known inducer of Id1 via Smad binding to consensus elements within the Id1 promoter. The findings above suggest that Src is required for Smad-mediated Id1 activation. To address this, we examined the effect of Src inhibitors on BMP-induced signaling and Id1 expression. In reporter assays, stimulation of A549 cells with BMP-2 (10 ng/mL; 1 h) induced a 3.4-fold increase in Id1 promoter activity above basal, serum-stimulated activity (Fig. 4A). Incubation with PP2 (10  $\mu$ mol/L) or AZD0530 (1  $\mu$ mol/L) reduced BMP-mediated Id1 promoter activity by 40% or completely blocked the response, respectively. Chromatin immunoprecipitation (ChIP) of unstimulated and BMP-stimulated A549 cells was performed using Smad1/5 antibody followed by DNA extraction and PCR using primers spanning the newly identified Src-responsive region of the Id1 promoter (Fig. 4B). BMP-2 (10 ng/mL; 1 hour) markedly induced Smad binding to the Src-responsive region of the Id1 promoter, and this process was completely blocked in the presence of PP2 (10  $\mu$ mol/L) or AZD0530 (1  $\mu$ mol/L). Consistently, BMP-induced Smad1/5 phosphorylation and Id1 expression was inhibited by PP2 and AZD0530 (Fig. 4C). This experiment also suggested Src activation by BMP-2, and indeed, a separate experiment confirmed that BMP-2 increased pY419-Src levels in a dose-dependent manner (Fig. 4D). Analysis of the blots with Scion Image software (Scion Corp.) and normalization for total Src revealed an average increase of the pY419-Src levels by 26% to 28% compared with baseline (data not shown).

To determine a physical interaction between Src and Smad, and to confirm the findings in another cell line, H460 lung cancer cells were stimulated with BMP-2 (10 ng/mL) for 30 or 60 minutes followed by immunoprecipitation of Src and Western blotting for Smad1/5 and Src (Fig. 5A). The amount of Smad1/5 present in immune complexes was increased in protein lysates from cells stimulated with BMP-2 (10 ng/mL; 1 hour), and this was blocked in the presence of AZD0530 (1  $\mu$ mol/L). Interestingly, a small (3–5 kDa) shift in the size of the Smad protein band occurred by stimulation with BMP-2, suggesting that phosphorylated Smad is recruited into the complex with Src. Next, the effect of Src inhibition on Smad1/5 nuclear translocation was studied. A549 cells were grown on chamber slides and incubated for 23 hours with DMSO, PP2 (10  $\mu$ mol/L), or AZD0530 (1  $\mu$ mol/L). Cells were then stimulated with BMP-2 (10 ng/mL; 1 hour). Immunofluorescent staining for Smad1/5 was performed and Hoechst dye used to visualize the nuclei (Fig. 5B). In unstimulated cells, Smad1/5 localization was primarily cytoplasmic. Although BMP-2 induced prominent nuclear accumulation of Smad1/5 in ~30% of the cells, which was consistent with a previous report (40), PP2 and AZD0530 almost completely blocked Smad nuclear translocation. In summary, the data showed the existence of cross-talk between Src and Smad pathways, and that Src is involved in BMP-2-mediated Smad activation and nuclear translocation.

**Involvement of Id1 in cancer cell invasion.** The cellular consequence of the Src-Id1 interaction was investigated by determining the effects of Id1 modulation on the invasiveness of A549 lung carcinoma cells. First, the efficacy of different methods of Id1 and Src antagonism to inhibit invasion was examined (Fig. 6A). Id1 siRNA reduced invasion by 50%, whereas PP2



**Figure 6.** Involvement of Id1 in lung cancer cell invasion. **A**, Matrigel invasion assays were performed in triplicates using A549 cells incubated for 24 h with control si RNA, Id1 siRNA (100 nmol/L), DMSO, PP2 (10  $\mu$ mol/L), or AZD0530 (1  $\mu$ mol/L). After 24 h, cells in the top chambers were removed, and cells in the bottom chambers were fixed with formaldehyde, stained with methylene blue, and counted. **B**, Id1 overexpressing A549 cells (*Id1*) and empty vector control cells (*vector*) were generated by retroviral infection and Geneticin selection as described under Materials and Methods. Western blotting was performed to determine the levels of Id1 and actin. Next, Matrigel invasion assays were performed in triplicates (**C** and **D**). After 24 h, cells in the upper chambers were removed, and cells in the bottom chambers were fixed with formaldehyde, stained with methylene blue, and counted (*bars*, 50  $\mu$ m).

(10  $\mu$ mol/L) and AZD0530 (1  $\mu$ mol/L) reduced invasion by 68% and 73%, respectively. Next, the dominant role of Id1 in conferring a more aggressive phenotype was tested by generating Id1-overexpressing A549 cells (A549-Id1), using retroviral gene transfer. Western blotting confirmed that Id1 expression was increased markedly in A549-Id1 cells compared with empty vector control cells (Fig. 6B). Matrigel assays revealed that Id1 overexpression enhanced invasion at 24 hours by 7.2-fold compared with vector control (Fig. 6C and D). These results confirmed the involvement of Id1 in lung cancer cell invasion.

## Discussion

The connection between BMP-2, Smad1, Id1, and cancer cell invasion is well-established. BMP-2 was found to be overexpressed in primary human lung cancer compared with normal tissue (18) and was shown to activate Smad1/5, to increase Id1 expression, and to promote invasion in lung cancer cells (40, 41). Critical regulatory elements in the Id1 promoter include a BMP-responsive region, a serum-responsive region, and a region associated with constitutive expression in breast cancer cells (14–16, 34). Consistent with previous data from noncancerous cells, the present

study implicates the crosstalk of Src tyrosine kinase signaling with the BMP-Smad pathway as an additional regulator of Id1 expression in cancer (42–45). Importantly, this was also associated with a substantial diminution in invasion. This was shown by the ability of multiple methods of Src inhibition (i.e., small-molecule antagonists, peptide inhibitors, and siRNA) to markedly reduce Id1 expression and promoter activity. Conversely, transient, enforced expression of a constitutively active Src mutant induced the Id1 promoter signal independently of serum or BMP-2. Using a series of Id1 promoter deletion constructs, we identified a novel Src-responsive region in the human Id1 promoter. This region contains the Smad-binding motif CAGC, and we provided evidence for the binding of Smad1/5 to the Src-responsive region. Consistent with the demonstration of a functional interaction between Src and BMP-Smad1/5, and temporal association of BMP-mediated activation of Src and Smad1/5, the formation of a signaling complex of Src and Smad1/5 was shown by coimmunoprecipitation experiments. Further work is expected to reveal the molecular mechanisms by which Src is recruited to the BMP receptor complex and is activated in response to BMP signaling, and by which Src may activate Smad1/5. In this context, previous work by others showed that PP1 (and to a lesser extent, PP2) significantly inhibited TGF $\beta$  receptor kinase activity and blocked subsequent Smad2/3 signaling; this suggested that some effects seen in our study may have resulted from direct inhibition of TGF $\beta$  receptor kinase by the small-molecule kinase inhibitors used (46). We used several different molecular approaches to Src targeting (RNA interference, small molecules, dominant-negative mutant, and inhibitory peptides) and the consistent results make it unlikely that off-target effects account for the main observations. Supporting this view, other studies showed that TGF $\beta$  did not activate, but rather inhibited, Id1 expression (14, 29, 47). Taken together, these data suggest a model of balanced Id1 regulation in which BMP acts positively on Id1 transcription via Smad1/5 and TGF $\beta$  acts as negative regulator via Smad2/3. Aberrant activation of Src may shift the balance toward increased Smad1/5 signaling, resulting in Id1 overexpression. However, the present report also supports the

existence of other undefined mechanisms of Id1 regulation because HT-29 and HCT-116 cells lacked a positive association between Src activation and Id1 expression. Because missense mutations in Smad4 exist in some colorectal cancer cells, including HT-29, Id1 expression may be driven by Smad-independent pathways in these cells (48). In this regard, the observation that Src inhibition reduced Id1 levels in MDA-MB231 cells, which constitutively express high levels of Id1 in a serum-independent manner, is encouraging (34).

Our study has several clinical implications. First and most important, it points toward a new molecular mechanism of action for Src inhibitors and suggests the use of Id1 as a biomarker. In line with this notion are gene expression signatures in breast and lung cancer, which include both Id1 and Src, and which are associated with tumor aggressiveness and responsiveness to Src inhibition, respectively (49, 50). Second, we speculate that therapeutic strategies based on Src inhibitor-mediated Id1 down-regulation may reduce tumor recurrence, angiogenesis, and metastasis. Beyond this, based on the implication of Src and BMP in osteogenesis, the findings described in the present report may lead to advances in the biology and treatment of malignant and nonmalignant bone disease.

## Acknowledgments

Received 11/27/2007; revised 2/5/2008; accepted 2/8/2008.

**Grant Support:** The Swiss Foundation for Medical-Biological Grants/Swiss National Science Foundation (1184/PASMA-108925; O. Gautschi) and the Swiss Cancer League/Oncosuisse (BIL OCS 01949-08-2006).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank AstraZeneca Pharmaceuticals (Macclesfield, UK) for AZD0530, Dr. Kit Lam (University of California Davis Cancer Center, Sacramento, California) for the Src-blocking peptides, and Dr. Don J. Fujita (Department of Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada) and Dr. June Zhou (UC Davis Cancer Center) for the Src expression constructs; Dr. Joy Yang (Department of Urology, UC Davis) and Ryan R. Davis (Gene Expression Resource, UC Davis Cancer Center) for the technical assistance; and the University of California Davis Cancer Center Gene Expression Resource, supported by Cancer Center Support Grant P30 CA93373-01 from the National Cancer Institute, for the microarray analysis.

## References

- Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. *Oncogene* 2004;23:7906–9.
- Summy JM, Gallick GE. Treatment for advanced tumors: SRC reclaims center stage. *Clin Cancer Res* 2006;12:1398–401.
- Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RL. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat* 2006;97:263–74.
- Lee D, Gautschi O. Clinical development of SRC tyrosine kinase inhibitors in lung cancer. *Clin Lung Cancer* 2006;7:381–4.
- Yokota Y. Id and development. *Oncogene* 2001;20:8290–8.
- Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 1990;61:49–59.
- Sun XH, Copeland NG, Jenkins NA, Baltimore D. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol Cell Biol* 1991;11:5603–11.
- Lyden D, Young AZ, Zagzag D, et al. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* 1999;401:670–7.
- Desprez PY, Hara E, Bissell MJ, Campisi J. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. *Mol Cell Biol* 1995;15:3398–404.
- Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003;115:281–92.
- Belletti B, Prisco M, Morrione A, Valentinis B, Navarro M, Baserga R. Regulation of Id2 gene expression by the insulin-like growth factor I receptor requires signaling by phosphatidylinositol 3-kinase. *J Biol Chem* 2001;276:13867–74.
- Lasorella A, Nosedà M, Beyna M, Yokota Y, Iavarone A. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature* 2000;407:592–8.
- Ohtani N, Zebede Z, Huot TJ, et al. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature* 2001;409:1067–70.
- Korchynski O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 2002;277:4883–91.
- Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. *J Biol Chem* 2002;277:3176–85.
- Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* 2002;7:949–60.
- Kang Y, Chen CR, Massague J. A self-enabling TGF $\beta$  response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* 2003;11:915–26.
- Langenfeld EM, Calvano SE, Abou-Nukta F, Lowry SF, Amenta P, Langenfeld J. The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. *Carcinogenesis* 2003;24:1445–54.
- Ling MT, Lau TC, Zhou C, et al. Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). *Carcinogenesis* 2005;26:1668–76.
- Wilson JW, Deed RW, Inoue T, et al. Expression of Id helix-loop-helix proteins in colorectal adenocarcinoma correlates with p53 expression and mitotic index. *Cancer Res* 2001;61:8803–10.
- Schindl M, Oberhuber G, Obermair A, Schoppmann SF, Karner B, Birner P. Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. *Cancer Res* 2001;61:5703–6.
- Schindl M, Schoppmann SF, Strobel T, et al. Level of Id-1 protein expression correlates with poor differentiation, enhanced malignant potential, and

- more aggressive clinical behavior of epithelial ovarian tumors. *Clin Cancer Res* 2003;9:779–85.
23. Tournay O, Benezra R. Transcription of the dominant-negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene Egr-1. *Mol Cell Biol* 1996;16:2418–30.
  24. Tepper CG, Gregg JP, Shi XB, et al. Profiling of gene expression changes caused by p53 gain-of-function mutant alleles in prostate cancer cells. *Prostate* 2005; 65:375–89.
  25. Swarbrick A, Akerfeldt MC, Lee CS, et al. Regulation of cyclin expression and cell cycle progression in breast epithelial cells by the helix-loop-helix protein Id1. *Oncogene* 2005;24:381–9.
  26. Lin CQ, Singh J, Murata K, et al. A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. *Cancer Res* 2000;60:1332–40.
  27. Desprez PY, Lin CQ, Thomasset N, Simpson CJ, Bissell MJ, Campisi J. A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1. *Mol Cell Biol* 1998;18:4577–88.
  28. Perk J, Gil-Bazo I, Chin Y, et al. Reassessment of id1 protein expression in human mammary, prostate, and bladder cancers using a monospecific rabbit monoclonal anti-id1 antibody. *Cancer Res* 2006;66: 10870–7.
  29. Tang B, Yoo N, Vu M, et al. Transforming growth factor- $\beta$  can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. *Cancer Res* 2007;67:8643–52.
  30. Fong S, Itahana Y, Sumida T, et al. Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. *Proc Natl Acad Sci U S A* 2003;100:13543–8.
  31. Kamath JR, Liu R, Enstrom AM, Lou Q, Lam KS. Development and characterization of potent and specific peptide inhibitors of p60c-src protein tyrosine kinase using pseudosubstrate-based inhibitor design approach. *J Pept Res* 2003;62:260–8.
  32. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A* 2001;98:31–6.
  33. Simon P. Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* 2003;19:1439–40.
  34. Singh J, Murata K, Itahana Y, Desprez PY. Constitutive expression of the Id-1 promoter in human metastatic breast cancer cells is linked with the loss of NF-1/Rb/HDAC-1 transcription repressor complex. *Oncogene* 2002;21:1812–22.
  35. Tanaka A, Fujita DJ. Expression of a molecularly cloned human c-src oncogene by using a replication-competent retroviral vector. *Mol Cell Biol* 1986;6:3900–9.
  36. Navab R, Gonzalez-Santos JM, Johnston MR, et al. Expression of chicken ovalbumin upstream promoter-transcription factor II enhances invasiveness of human lung carcinoma cells. *Cancer Res* 2004;64:5097–105.
  37. Irby RB, Malek RL, Bloom G, et al. Iterative microarray and RNA interference-based interrogation of the SRC-induced invasive phenotype. *Cancer Res* 2005;65: 1814–21.
  38. Chuan YC, Pang ST, Cedazo-Minguez A, Norstedt G, Pousette A, Flores-Morales A. Androgen induction of prostate cancer cell invasion is mediated by ezrin. *J Biol Chem* 2006;281:29938–48.
  39. Gordon LA, Mulligan KT, Maxwell-Jones H, Adams M, Walker RA, Jones JL. Breast cell invasive potential relates to the myoepithelial phenotype. *Int J Cancer* 2003;106:8–16.
  40. Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. *Oncogene* 2006;25:685–92.
  41. Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein-2-induced transformation involves the activation of mammalian target of rapamycin. *Mol Cancer Res* 2005;3:679–84.
  42. Wong WK, Knowles JA, Morse JH. Bone morphogenetic protein receptor type II C-terminus interacts with c-Src: implication for a role in pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2005;33:438–46.
  43. Mima A, Matsubara T, Arai H, et al. Angiotensin II-dependent Src and Smad1 signaling pathway is crucial for the development of diabetic nephropathy. *Lab Invest* 2006;86:927–39.
  44. Kersten C, Dosen G, Myklebust JH, et al. BMP-6 inhibits human bone marrow B lymphopoiesis-upregulation of Id1 and Id3. *Exp Hematol* 2006;34:72–81.
  45. Hagen M, Fagan K, Steudel W, et al. Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1473–9.
  46. Maeda M, Shintani Y, Wheelock MJ, Johnson KR. Src activation is not necessary for transforming growth factor (TGF)- $\beta$ -mediated epithelial to mesenchymal transitions (EMT) in mammary epithelial cells. PP1 directly inhibits TGF- $\beta$  receptors I and II. *J Biol Chem* 2006;281:59–68.
  47. Murillo MM, del Castillo G, Sanchez A, Fernandez M, Fabregat I. Involvement of EGF receptor and c-Src in the survival signals induced by TGF- $\beta$ 1 in hepatocytes. *Oncogene* 2005;24:4580–7.
  48. Woodford-Richens KL, Rowan AJ, Gorman P, et al. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. *Proc Natl Acad Sci U S A* 2001;98:9719–23.
  49. Minn AJ, Gupta GP, Padua D, et al. Lung metastasis genes couple breast tumor size and metastatic spread. *Proc Natl Acad Sci U S A* 2007;104:6740–5.
  50. Bild AH, Yao G, Chang JT, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006;439:353–7.

**A novel neuropeptide-autocrine model for androgen-insensitive prostate  
cancer: aberrant activation of androgen receptor and  
inhibition by AZD0530<sup>1</sup>**

**Joy C. Yang, Joon-ha Ok, J. Erik Busby, Alexander D. Borowsky, Hsing-Jien Kung, and  
Christopher P. Evans<sup>2</sup>.**

Departments of Urology (J.C.Y., J.-h.O., J.E.B., and C.P.E.) Biological Chemistry (H.-J.K.) and  
Cancer Center (H.-J.K., C.P.E.), University of California at Davis, Sacramento, CA 95817, and  
Center for Comparative Medicine, University of California, Davis, CA 95616 (A.D.B.)

<sup>1</sup>Supported by NIH Grants KO8 DK60748-01, 2RO1 DK/AG52659- 04, and Department of  
Defense PC10520. Mention of trade name, proprietary product or specific equipment does not  
constitute a guaranty of warranty by the Department of Defense, nor does it imply approval to  
the exclusion of other products. The views expressed herein represent those of the authors and  
do not necessarily represent the position of Department of Defense.

<sup>2</sup>To whom requests of reprints should be addressed, at the Department of Urology, University of  
California, Davis School of Medicine, 4860 Y St., Suite 3500, Sacramento, CA 95817.

Key words: GPCR, neuroendocrine, neuropeptides, androgen-independence, Src kinase, prostate  
cancer

## **ABSTRACT**

Neuroendocrine differentiation of CaP cells is often detected under androgen-deprived conditions. Neuropeptides released by these neuroendocrine-differentiated CaP cells may facilitate the development of androgen independence. Exemplified by GRP (gastrin-releasing peptide), these neuropeptides transmit their signals through G-protein coupled receptor (GPCRs), which are often overexpressed in prostate cancer. We developed an autocrine neuropeptide model by overexpressing GRP in LNCaP cells to attain their androgen-independence. LNCaP-GRP cells were evaluated for proliferation, migration and tumorigenesis in androgen-free environments in vitro and in vivo. LNCaP-GRP cells orthotopically implanted in castrated nude mice produced significant tumors, with expression of GRP, prostate-specific antigen, and androgen receptor nuclear localization. Chromatin immunoprecipitation studies of LNCaP-GRP clones suggest that expressed GRP signals, activates and recruits AR to the cognate promoter in the absence of androgen. Recultured LNCaP-GRP xenografts (GRP-Pro cells) showed enhanced androgen independent growth and motility. A Src family kinase (SFK) inhibitor, AZD0530 inhibits not only androgen-independent growth and migration but also AR nuclear translocation of the GRP and GRP-Pro cell lines, demonstrating its potential in the treatment of hormone refractory CaP. In vivo study showed AZD0530 profoundly inhibits tumor metastasis in severe combined immunodeficient (SCID) mice implanted with GRP-Pro cells. This xenograft model demonstrates autocrine, neuropeptide- and Src kinase-mediated progression of androgen-independent CaP post-castration, and is potentially useful for testing novel therapeutic agents.

## **INTRODUCTION**

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer deaths (1). Androgen withdrawal initially induces apoptosis and cell

cycle arrest in CaP; however, CaP eventually loses its dependence on androgens and progresses to an androgen-independent state. Various mechanisms have been postulated to account for the conversion of CaP into hormone-refractory state, including the aberrant activation of androgen receptor by peptide growth factors and ligands for GPCRs (2-4). If true, these mediators and components of their signal pathways are potential targets for therapeutic intervention of hormone-refractory CaP. It has been reported that androgen withdrawal from androgen-dependent CaP cells (5) or treatment with stimuli such as IL-6 and forskolin in vitro promotes acquisition of the neuroendocrine phenotype through transdifferentiation (6). Cumulative evidence suggests that neuroendocrine differentiation of CaP may be a cofactor involved in tumor progression and androgen independence (7).

Neuroendocrine cells are identified by their neurosecretory granules and expression of neuron specific markers including chromogranin A, neuron-specific enolase and mitogenic neuropeptides such as bombesin/GRP, somatostatin, calcitonin, and parathyroid hormone-related peptides (7). Neuropeptides have been identified as potent paracrine and autocrine growth factors in human cancers to include lung, gastrointestinal, pancreatic, brain, and prostate (8-13). In prostate cancer, previous studies by others and by us have shown that neuropeptides promote cell growth (14), migration and protease expression (15) in PC-3 cells, and androgen-independence in LNCaP cells (3, 5). Androgen independence in CaP patients is shown to correlate well with elevated serum levels of chromogranin A (16). Elevated expression of GRP receptors are often detected in CaP specimens (17, 18).

The bombesin/GRP family is among the most studied neuropeptide group in CaP. Bombesin/GRP transduces signals by engaging heterotrimeric G protein-coupled receptors located on the cell surface (19). Upon binding to its receptors, bombesin/GRP elicits calcium mobilization in PC-3 and DU 145 cells (20, 21) and promotes growth and cell invasiveness via

proteolytic activities of MMP's in LNCaP and PC-3 cells (15). We have previously shown that exogenous bombesin/GRP activates AR and supports androgen-independent growth in LNCaP through signaling mediated by non-receptor tyrosine kinases such as Src, FAK and Etk (3). In vivo androgen withdrawal following establishment of LNCaP tumors results in increased neuroendocrine cells (5). Together, these data suggest that castration induced neuroendocrine differentiation may release soluble factors, which sustain the growth and survival of androgen-deprived cells, contributing to tumor androgen-independence and metastasis.

In this paper, we describe a neuropeptide xenograft model and use it to test inhibition of the tyrosine kinase pathway implicated in the development of androgen-independence. We introduced the GRP-expressing vector into LNCaP cells to establish an autocrine neuroendocrine model. The GRP clones exhibited enhanced proliferation and migration properties under androgen-depleted conditions, and developed significant tumors in castrated nude mice, providing evidence for GRP's role in androgen-independent growth through modulation of AR. We tested the effect of a SFK inhibitor AZD0530 on recultured xenograft cells both in vitro and in vivo. Our results showed that AZD0530 effectively blocked the androgen-independent growth and migration of LNCaP cells mediated by autocrine GRP, through inhibiting activation of the Src/FAK/Etk complex. SCID mice implanted with GRP-autocrine LNCaP cells and treated with AZD0530 showed a complete inhibition of tumor metastasis.

## **MATERIALS AND METHODS**

**Cell culture.** LNCaP cells (ATCC, passages 38-43) were kept in RPMI1640 with 10% regular FBS. When stimulated, cells were switched to phenol-red free RPMI1640 with 5% charcoal-stripped androgen-free (CS) serum.

**Proliferation assays.** Cells were grown in CS medium alone or with supplemented with 100 nM of bombesin, with 1 µg/ml of bombesin/GRP specific monoclonal antibody 2A11 (22), 5 µM of GRP receptor antagonist RC3095 (23) or transfection of 100 µM of small inhibitory RNA (siRNA, sense sequence GGAAACAGUUCAACACUCAUU, validated by RT-PCR for inhibition, Dharmacon) for GRP receptor. Cells were trypsinized and counted by trypan blue exclusion method after 48 hours or over 6 days for siSrc transfection.

**Chemotaxis migration assay.** Migration assays were performed in a Boyden chamber with 8 µm Nucleopore membrane coated with human plasma fibronectin (50 µg/ml).  $2 \times 10^4$  LNCaP cells were placed in the upper wells with testing agents in the lower wells, and incubated at 37°C for 4 hours to allow cell migration. At the end of incubation, the membrane was stained by Diff-Quik Stain Kit and mounted on microscopic slides for counting. Each experiment was performed in triplicate. AZD0530 (500 nM) or siSrc transfection was used for inhibitor studies.

**GRP-expressing construct and transfection.** GRP cDNA was amplified from the small cell lung carcinoma DMS53 cell line (ATCC), which expresses GRP. The amplified cDNA was inserted into mammalian expression vector pcDNA3.1-Zeocin (Invitrogen). LNCaP cells were transfected with this GRP construct or the empty vector and stable transfectants were selected with Zeocin (100µg/ml) as GRP or Zeo (the mock transfectant). Presence of the GRP gene in selected clones was confirmed by Northern blotting and RT-PCR.

**Quantitation of secreted GRP.** CM collected from LNCaP, LNCaP-zeo, GRP1-1, GRP4-9 and DMS53 cells was concentrated by passing through PrepSep C18 reverse columns (Fisher) and the retentate was eluted with acetonitrile:water:acetic acid (59:40:1) mixture (24). The reconstituted solvent-free eluents were assayed for bombesin/GRP with a Bombesin EIA kit (Peninsula Lab).

**Soft Agar Assay.**  $2 \times 10^4$  cells were plated in the midst of 0.3% agar in CS medium with or without 2A11 (1  $\mu$ g/ml). Colony formation was examined after 4 weeks. For the recultured GRP xenograft bicalutamide (5  $\mu$ M) was used additionally.

**In vivo tumor biology.** Animal studies were conducted in accordance with institutional ethical guidelines for the care and use of experimental animals. Surgical castration was performed and immediately following castration,  $2 \times 10^6$  LNCaP-Zeo and GRP cells co-suspended with 30% matrigel were injected orthotopically into twenty and twelve castrated nude mice, respectively. At the end of 4 months, mice were sacrificed and their prostates were collected for pathological analyses. Tumor sections were immunohistochemically stained with antibodies for GRP (RGG7130, Peninsula Laboratories), AR (PG21, Millipore) and PSA (ER-PR8, Dako) and detected using the DAKO Envision+ Kit. PSA levels were determined by the Micro PSA ELISA kit (Fitzgerald Industries).

Tumors were dissociated into single-cell suspensions with collagenase and plated in CS media. The derived LNCaP GRP sublines termed GRP-Pro (derived from Prostate) were pooled together and subjected to soft agar assay to examine their androgen-independent and tumorigenic characteristics.

For inhibitor study, fourteen castrated SCID mice were orthotopically implanted with  $4 \times 10^6$  re-cultured GRP-Pro cells. SCID mice were used to better study tumor metastasis. Two weeks after surgery, mice are divided into two groups, with 7 treated with 50mg/kg/day via esophageal gavaging (AZD0530-treated) and 7 with buffer only (control). The study was terminated when one of the control mice succumbed to tumor burden. All the mice were euthanized, their primary tumors excised for weighing and IHC staining with p-Src (Cell Signaling), p-FAK (ABR) or AR antibodies and lymph nodes examined for metastasis.

**Transient transfection assays.** Zeo, GRP4-9 and GRP Pro cells were seeded in 24-well plates, transfected with 0.2 µg of PSA-Luc (promoter region, 630 bp) with the internal control pTK-RL using Effectene<sup>®</sup> (Qiagen). Transactivation was examined by the dual-luciferase assay (Promega). For RNA interference, standard siCONTROL (D-001210-02, SC) and on-target plus SMART pool human Src (L-003175-00, SiSrc, Dharmacon) were complexed with Lipofectamine 2000 (Invitrogen) and delivered to cells grown in CS media at a final concentration of 100 nM.

**Chromatin Immunoprecipitation.** LNCaP-Zeo, GRP, and GRP-Pro cells grown to sub-confluency were switched to CS media for 3 days. Treatment with R1881 was performed 6 hours before harvesting. Chromatin immunoprecipitation was performed as described (4, 25, 26) with 6 µg of anti-AR antibody (PG-21, Millipore). Standard PCR cycling protocol was performed with 58°C for annealing for 30 cycles. Primers for AR enhancer region are: 5'catgttcacattagtagcaccttg3' and 5'tctcagatccaggcttgcttac3'; for proximal ARE region: 5'tctctgagtgcgtgtgtcttag3' and 5'agccctataaaacettcatcc3'; and for intervening region: 5'tcatccactcatcatccagcatc3' and 5'ggagagcaatagacttgggaacc3'.

**Immunofluorescent staining of AR.** Cells (2,500) were plated in 4-well chamber slides in CS media a day before fixing with 2% paraformaldehyde for staining. Anti-AR (N-20, Santa Cruz) and anti-rabbit Alexa Fluor 647 (Invitrogen) were used as the primary and secondary antibodies for staining, respectively. Immunofluorescent cells were visualized using an Olympus BX61 motorized reflected fluorescence microscope system with an AMCA filter for DAPI and a Cy5 filter for Alexa Fluor647 using the SlideBook4.1 software (Intelligent Imaging Innovations).

**Immunoprecipitation and Western blot.** LNCaP-Zeo, GRP and GRP-Pro cells were subjected to androgen withdrawal for 3 days with or without exposure to AZD0530 (1 µM). Cell lysates were collected in IP buffer containing proteinase and phosphatase inhibitors, incubated with anti-FAK and subsequently protein G agarose beads for immunoprecipitation.

Phosphorylation of the respective precipitated proteins was detected by anti-p-Src family (Cell Signaling), p-FAK (Invitrogen) and p-Etk (Cell Signaling) antibodies after Western blotting analysis. Signals were detected by ECL system (Amersham) followed by exposure to X-ray film.

**Statistics.** All in vitro data were from at least three independent experiments and subjected to paired t-tests using Statview program.

## RESULTS

It has been shown that bombesin confers androgen-independent growth of LNCaP cells (3). We validated that bombesin signals through the GRP receptor with specific inhibitors such as bombesin/GRP specific monoclonal antibody 2A11 and GRP receptor antagonist RC3940-II. Bombesin also stimulated cell migration as compared to the negative control (supplementary data #1).

**Expression of GRP enhances proliferation and migration of transfected LNCaP cells.** We established an autocrine model by introducing a GRP overexpressing vector to androgen-sensitive LNCaP cells to study the signaling pathways involved in androgen independence in vitro and in vivo. Stable LNCaP-GRP transfectants were established by overexpressing GRP cDNA and screened by Northern blotting and RT-PCR (Figure 1A). Positive clones (e.g. GRP1-1 and GRP4-9) were isolated and characterized. Bombesin/GRP enzyme immunoassay performed on CM collected from parental LNCaP, the vector-transfected control LNCaP-zeo, GRP1-1 and 4-9, as well as the GRP expressing DMS53 cells confirmed GRP expression in the two GRP clones (Figure 1B). GRP1-1 and 4-9 cells produce almost 5 fold more GRP than the control lines, but comparable to DMS53 cells. Antibody 2A11, GRP receptor antagonist RC3905 and siRNA for the GRP receptor effectively inhibited the androgen-

independent growth of GRP1-1 and 4-9 to 20-60% of the control (Figure 1C). Negative control using siRNA targeting green fluorescence protein showed no effect on growth (data not shown). These data support the notion that GRP/bombesin is able to confer androgen-independent growth of LNCaP through binding to its membrane receptor. If the androgen independent growth is due to the autocrine release of GRP into the media, we would expect a chemotactic effect from GRP CM. As expected, LNCaP-Zeo migration was stimulated by bombesin (Figure 1D). GRP CM stimulated LNCaP-Zeo migration by more than 3-fold and this migration was significantly reduced by 2A11 ( $p \leq 0.001$ ), suggesting GRP's involvement. Migration of GRP1-1 and 4-9 towards ctrlCM was two-fold greater than that of LNCaP-zeo, and could be further stimulated by GRP CM, and significantly inhibited by 2A11 ( $p \leq 0.001$ ). These data showed that LNCaP-GRP cells release GRP, which confers androgen-independent growth and migration on themselves through autocrine loop as well as on the control LNCaP-Zeo cells.

**GRP promotes in vitro and in vivo tumorigenesis in androgen-free environments.**

Soft agar assay was performed to assess in vitro tumorigenicity. GRP1-1 and 4-9 produced significantly more colonies than LNCaP-Zeo in CS medium, suggesting that the autocrine GRP induces both androgen- and anchorage-independent growth (Figure 2A). 2A11 significantly inhibited colony formation of both GRP1-1 and 4-9 ( $p \leq 0.05$  and  $p \leq 0.0005$ ). We then used the GRP clones for in vivo tumor study. Orthotopic prostatic implantation of GRP4-9 cells into prostates of castrated nude mice resulted in tumor growth in 8 of 12 mice. In contrast, 0 of 20 castrated mice implanted with LNCaP-zeo cells displayed any tumor growth. To generalize this finding, GRP1-1 was also orthotopically implanted and 4 of 5 mice produced tumors. H and E staining of the tumors showed characteristic human CaP tumors adjacent to normal mouse prostate tissue (Figure 2B). IHC staining (Figure 2C) showed staining of GRP (*a* and *b*) was evident throughout the cytoplasm of the tumor regions, yet minimally detected in the normal

mouse prostate epithelium of the tumor, despite the fact that the GRP antibody used reacts with both human and mouse GRP. Staining with anti- AR antibody (*c* and *d*) demonstrated its nuclear translocation in tumor cells, indicative of GRP ligand activation. PSA expression (*e* and *f*) was extensive in the tumor specimens, again supporting GRP-mediated AR activation. Mean serum PSA level in castrated LNCaP-GRP tumor mice was  $208.9 \pm 24.6$  ng/ml serum, as compared to  $6.13 \times 10^{-5}$  ng/ml in castrated LNCaP-zeo mice.

Tumors harvested from GRP implanted mice were re-cultured in vitro to establish a xenograft cell line, labeled GRP-Pro. Expression of PSA, AR and GRP in GRP-Pro cells was analyzed by RT-PCR analysis for the authenticity of the clones (supplementary data #2). RT-PCR for the endogenous PSA mRNA for all clones is shown in the supplementary data. Soft agar assay using GRP-Pro cells showed their aggressive nature as manifested by their androgen- and anchorage- independent growth in 2 weeks (Figure 3A). This growth was partially inhibited by 2A11 and the androgen inhibitor, bicalutamide, individually or in combination (with significant difference  $p \leq 0.05$ ) suggesting that growth is dependent on both GRP and AR.

**GRP modulates activation of the androgen receptor.** We further sought to illustrate GRP-mediated AR activation at the molecular level. Transactivation assay was performed with LNCaP-Zeo, GRP-4-9 and GRP-Pro cells in CS media using promoter PSA-Luc as the reporter. Expression of PSA-Luc in GRP4-9 and GRP-Pro is 1.8 and 4.5 fold higher than in LNCaP-Zeo cells (Figure 3B). This suggests GRP secreted from GRP cells is driving the expression.

Addition of synthetic androgen R1881 induced PSA-Luc expression in LNCaP-Zeo cells more than 6 fold, but much less in GRP4-9 and GRP-Pro cells probably because the GRP-activated AR, through post-translational modification, already adopted an active conformation and may not be further stimulated by R1881. If GRP activates AR in GRP-Pro cells, AR should be recruited to ARE sites in the PSA promoter. We therefore performed the ChIP assay on LNCaP-

Zeo, GRP4-9 and GRP-Pro cells in CS or CS+R1881 conditions. AR binding was analyzed by PCR using respective primers against enhancer (E) and proximal (P) ARE regions, and an intervening (I) region void of any ARE sites. Figure 3C shows AR binds to PSA P region in GRP4-9 and GRP-Pro even in the absence of androgen. When treated with R1881, AR binds preferentially to the E site in LNCaP-Zeo; whereas in GRP4-9 and GRP-Pro, AR binding was evenly detected at both P and E sites.

**Src and FAK tyrosine kinases play important roles in GRP-mediated androgen-independent growth and migration.** Exogenous bombesin induces AR nuclear translocation, and this induction is inhibited by Src inhibitor PP2 (25). In our LNCaP GRP mouse model, AR is localized to the nuclei as shown in the tumor IHC staining (Figure 2C). We further compared the GRP cells with the mock control by immunofluorescent staining to confirm AR nuclear localization in GRP cells through autocrine GRP-mediated activation (Figure 4). Staining of AR is limited to the cytoplasm in Zeo cells grown in CS media but concentrated to the nuclei of GRP cells (counted 65% nuclei with AR). This localization was inhibited by AZD0530, a selective SFK inhibitor demonstrating significant effects on prostate cancer cells (27). Almost half of GRP cells (35% nuclei with AR remaining) lost nuclear staining of AR when Src activity is inhibited. These data confirm that GRP activates AR through Src and promotes its nuclear translocation, consistent with recent data that Src directly phosphorylates AR at Y534 resulting in nuclear translocation (28).

Among all the tyrosine kinases expressed in LNCaP cells, we previously showed that Src and FAK are most prominently activated by bombesin (3). Activated Src and FAK engage Etk, a tyrosine kinase shown to be involved in prostate carcinogenesis (3, 29). Src and FAK form a complex through binding between the phosphorylated Y397 in FAK and the SH2 domain in Src (30), whereas FAK associates with Etk via the FERM domain of FAK and the PH domain of Etk

(31). These three kinases cross activate one another with increased tyrosine phosphorylation of the complex. Using AZD0530, we examined whether the androgen-independent growth and migration stimulation observed in our autocrine model is mediated through the Src/FAK signaling pathway. In LNCaP cells, in addition to Src, another member of SFK, Lyn, is also significantly expressed. We thus examined the phosphorylation status of Src, Lyn and FAK kinases in all cell lines grown in CS medium. We immunoprecipitated Src and Lyn proteins with their respective antibodies, then probed with anti-p-Src or anti-p-Y antibodies. For FAK, we used anti-p-FAKY861, residue phosphorylated by Src, which is another indicator of the activity of SFKs. All the GRP and GRP-Pro lines displayed higher levels of kinase phosphorylations compared to Zeo cells after exposure to CS serum for 3 days and the phosphorylations were inhibited by AZD0530 (Figure 5A). The data showed that 1) autocrine-GRP indeed activates the SFKs; and 2) AZD0530, a pan-Src inhibitor, effectively blocks the activity of Src family members. Thus, while in the ensuing studies we will focus on the molecular characterizations of Src, the biological effects observed are likely due to the combined inhibition of all SFKs expressed in LNCaP cells. We previously reported that when activated, Src forms a complex with FAK and Etk and these kinases cross activate one another. Co-immunoprecipitation of Src, FAK and Etk kinases with the anti-FAK antibody confirms the complex formation and showed elevated activation of the three kinases in GRP and GRP-Pro cells compared to Zeo cells. Treatment with AZD0530 significantly reduced the degree of tyrosine phosphorylation of all three kinases but to a much less extent, the association between FAK and Src. (Figure 5A).

Regarding proliferation, AZD0530 reduced GRP-Pro cell growth in a dose-dependent manner and inhibited the anchorage- and androgen-free growth of GRP-Pro cells (supplementary data #3). To ensure AZD0530 targets Src through which GRP mediates AR activation, RNA

interference experiment for Src (siSrc) was performed. Transfection of siSrc into GRP4-9 and GRP-Pro cells greatly impaired their ability to grow in CS media compared to their respective non-target controls (SC, scramble RNA); whereas the LNCaP-Zeo cells do not grow well in the androgen-deprived condition with or without siSrc (Figure 5B). These data support that Src is a major target in neuropeptide-mediated AR activation, possibly through its downstream kinases such as FAK and Etk. Both FAK and Etk function in cell adhesion and migration, and inhibition of Src would reduce LNCaP-GRP and GRP-Pro cell migration. As a result, motility of GRP4-9 ( $p \leq 0.05$ ) and GRP-Pro ( $p \leq 0.0005$ ) cells was significantly inhibited by AZD0530 (500 nM) (Figure 5C). Knocking down Src with siSrc transfection into GRP4-9 and GRP-Pro cells also reduced cell migration to the comparable level as Zeo cells. These data support the notion that the GRP-mediated androgen-independent growth and migration is principally through SFK, especially Src kinase.

**SFK inhibitor AZD0530 prevents tumor metastasis in SCID mice.** With the encouraging results of AZD0530 inhibition in vitro, we evaluated it in our orthotopic GRP mouse model. Fourteen castrated SCID mice implanted with GRP-Pro cells; half of them were administered 50 mg/kg/day of AZD0530 (treatment) beginning two weeks after surgery (to permit tumor establishment) and half with buffer only (control) for eight weeks. All control animals grew tumor with lymph node metastasis (Figure 6A). H & E staining (insert) of the lymph node validated its human prostate cancer origin. Five of seven treated animals produced primary tumors, but none had metastasis. IHC staining using anti-p-Src and anti-p-FAK antibodies showed reduced phosphorylation levels in the treatment samples (Figure 6B) confirming the effect of AZD0530 in tumors. When probed with anti-AR antibody, the control tumor showed AR nuclear localization as in Figure 2C. AR staining became undetectable in AZD0530 treated tumor since castrated animals were used. As a result, PSA levels from sera of

AZD0530 treated mice showed significant reduction ( $p=0.02$ ) compared to controls (Figure 6C). Primary tumor sizes in the treated animals were smaller, although not statistically significant ( $p=0.104$ ) when compared to control animals. AZD0530 however completely blocks tumor metastasis possibly through inhibiting SFK and FAK.

## DISCUSSION

In this study, we report the development of a neuropeptide-autocrine model for androgen-insensitive CaP. This model was not designed to study neuroendocrine tumors of prostate, which are relatively rare, but to study the effect of neuropeptides released from neuroendocrine prostate cells on CaP progression following androgen ablation. There is abundant literature documenting the correlation of increased number of post-mitotic neuroendocrine cells with the development of castration-resistant CaP and reports showing overexpression of neuropeptides and neuropeptide receptors in advanced CaP (16-18). Yet, the biological effect of neuropeptides on CaP has not been clearly demonstrated. We present in vitro and in vivo data that the GRP autocrine loop is sufficient to establish androgen independence in LNCaP cells by inappropriate activation of the androgen receptor. We also show that GRP activates Src, Lyn, FAK and Etk tyrosine kinases, which confer motility and invasiveness to CaP. Our in vivo inhibitor study demonstrates that administration of Src inhibitor AZD0530 completely blocks tumor metastasis in the androgen-independent environment.

There are numerous reports on growth factors (32), cytokines, chemokines (2, 4) and neuropeptides (3, 25) promoting androgen-independent growth of LNCaP cells. While the ligands inducing AR activation are different, many of them transmit signals through SFK (3, 4, 25). In the present model, we focused on neuropeptides which are coupled to GPCRs and as we showed before, activate the tyrosine kinase complex Src/FAK/Etk (3). We hypothesized that induced expression of GRP in LNCaP cells may facilitate a more aggressive phenotype via

autocrine stimulation. Our engineered LNCaP GRP cells demonstrated androgen- and anchorage-independent growth and superior migration compared to control LNCaP-Zeo cells, and the bombesin/GRP specific antibody 2A11 partially inhibited the increased growth and migration. This incomplete inhibition by 2A11 may be due to secretion of other neuropeptides such as neurotensin from the GRP clones (data not shown). These other factors also activate GPCRs, thus there is greater inhibition with GRP receptor inhibition compared to 2A11. Consistent with the in vitro properties, autocrine GRP activity supports androgen-independent tumorigenesis of LNCaP-GRP clones in castrated mice. IHC staining demonstrated nuclear localization of AR and PSA expression in tumor cells, supporting GRP stimulation of AR in the absence of testicular androgens, which is the sole source of androgen in mice. These observations build upon those reported by Burchardt and colleagues who demonstrated that androgen withdrawal of established in vivo LNCaP tumors resulted in enrichment of neuroendocrine cells (5). Herein we demonstrate that Src mediates the nuclear-translocation and target recruitment of AR induced by GRP, based on in vitro (ChIP assay) and in vivo (tumor IHC) analyses. A related report using a neuroendocrine mouse prostate allograft also showed neuroendocrine secretions were sufficient to support androgen-independent growth of LNCaP and PSA expression in vivo (33). These data together firmly establish the potential of neuropeptides secreted by neuroendocrine differentiated cells to induce androgen independence, and this process involves Src activation.

Elevated tyrosine phosphorylations, especially Src activation were shown in hormone-refractory prostate cancer xenografts derived from castrated animals (28). In this study, we showed that Src (and likewise, Lyn) is activated both in the free form as well as in the Src/FAK/Etk complex form. As expected, FAK and Etk are also activated as indicated by their heightened phosphorylation status. Impressively, AZD0530 treatment completely blocked these activations. The exact mechanism how bombesin/GRP activates AR to induce androgen

independent growth of LNCaP is not fully understood. Although GRP has been reported to mediate MAPK and Src activation through epidermal growth factor receptor (EGFR) in some human malignancies (34), we observed no increased tyrosine phosphorylation of EGFR in LNCaP cells upon bombesin stimulation (data not shown). Despite reports implicating Src kinase in the development, growth, progression and metastasis of human cancers, only one report correlates elevated Src activation and AR phosphorylation to hormone-refractory CaP (28). This report elegantly showed that tyrosine residue Y534 of AR is the direct target of Src phosphorylation, which effectively translocates AR into the nucleus for gene transcription in the absence of androgen. Another report relates expression of a truncated c-kit tyrosine kinase, which is a strong activator of Src, to advanced stages of CaP (35), suggesting the importance of Src activity in CaP progression. Here we show that reversion of androgen-independent growth of GRP lines by knocking out Src with siRNA supports a significant role for Src in GRP-mediated cell proliferation. It is speculated that modification of AR or its co-activators by phosphorylation (36) or acetylation (37) mimics the conformation change caused by androgen binding to activate AR in the absence of its cognate ligand. Src may potentially phosphorylate AR directly or through an intermediate molecule (28). Yet, since no tyrosine-phosphorylated AR was detected in bombesin-treated LNCaP cells (25), the exact mechanism how Src is involved still remains to be elucidated. In the ChIP assay, GRP mediated AR recruitment preferentially to the proximal ARE site in the PSA promoter, rather than to the enhancer ARE. This observation may reflect conformational modification of AR by Src or a downstream kinase, which facilitates AR activation by assembling a different co-activator complex to elicit gene transactivation in the absence of its natural ligand. Similarly, the reason why addition of R1881 to GRP clone did not increase the reporter activity further may be that GRP-activated AR is already in active conformation and may not be further stimulated by androgen. Our studies also

revealed that post-translationally activated AR may be conformationally different from ligand bound AR, a finding supported by previous studies (4, 25). Further structural analysis will be required to substantiate this notion.

LNCaP cells are usually not very migratory, but overexpression of GRP under androgen-free conditions enhances LNCaP-GRP cell migration. Another reported mechanism is that bombesin activates RhoA and Rho-associated coiled-coil forming protein kinase to promote CaP cell migration and invasion (38). Since RhoA can be activated by Etk (39) which is activated by Src (40), our data are consistent with their findings. FAK phosphorylation in bombesin-stimulated PC-3 cells is linked to cell motility and invasion (41). In collaboration with FAK, Etk is also involved in integrin signaling and promotes PC-3M migration (31). Knocking down Etk expression with its specific siRNA inhibits LNCaP cell proliferation (29, 42), and prostates from Etk transgenic mice exhibit pathological changes resembling human prostate intraepithelial neoplasia (29). Complexing of these three kinases results in synergistic activation and may transduce GRP modulated signaling in CaP cell proliferation, migration and survival.

Targeting the bombesin/GRP receptor for cancer therapy is undergoing early clinical trials (43). Other clinical trials have reported promising results using tyrosine kinase inhibitors in cancer therapy; for instances, imatinib (Gleevec, STI571) for chronic myelogenous leukemia and gastrointestinal stromal tumors (44, 45) and trastuzumab (Herceptin, Her-2 antibody) for breast cancer (46). Our approach suggests using a SFK inhibitor to target the activation of non-receptor tyrosine kinases. Through inhibiting Src, AZD0530 prevents the Src-specific activation of FAK, AR and possibly Etk and effectively blocks tumor metastasis in our GRP autocrine model. Complex growth factors available in tumor microenvironments and the compensatory pathways involving cell proliferation downstream to Src may be factors why AZD0530 alone could not halt primary tumor growth.  $IC_{50}$ 's for inhibiting FAK, paxillin and P130Cas

responsible for migration were 4-64 fold lower than those for cyclin-D1 and c-Myc for proliferation (27). AZD0530 has been tested in tamoxifen-resistant breast cancer cells to suppress tumor cell migration through modulating FAK (47). Treating A549 lung carcinoma cells with AZD0530 results in down regulation of Id1 gene expression possibly through BMP-Smad-Id pathway involved angiogenesis and metastasis (48). The other small molecule Src inhibitor Dasatinib (49), displays similar inhibitory mechanism as AZD0530 with more inhibition on metastasis than tumor growth in vivo (50). Lyn, a member of SFK, found to play a role in PC-3 tumor progression, was also inhibited by AZD0530 (data not shown).

In addition to neuropeptides, we have previously shown Src kinase activation as central to IL-8-induced androgen-independent prostate cell growth (4). Importantly, IL-8 is also a ligand for GPCRs. As such, inhibition of signaling transduction through Src kinase as a downstream target may block the oncogenic stimulation for more than one ligand. The specific mechanisms activating AR remain to be elucidated, but the pathways identified suggest Src kinase inhibition may prove useful in the treatment of androgen-independent CaP.

## **ACKNOWLEDGEMENTS**

We thank Dr. Cuttitta (NIH) for providing the monoclonal antibody to bombesin, 2A11, and Dr. Schally (Tulane University) for GRP receptor antagonists RC3095 and RC3940-II. The work is supported by NIH grant KO8-DK60748-01 (CPE), RO1-DK52659 (HJK), DOD PC060508 (HJK) and DOD grant PC040161 (CPE).

## **Figure legends**

Figure 1. The model of an androgen-independent GRP expressing prostate cancer line, with evidence of enhanced proliferation and migration: *A*, Northern blot and RT-PCR assays verified expression of GRP gene into LNCaP GRP clones compared to the parental LNCaP / mock-transfected Zeo cells (negative controls) and DMS53 (positive control) cells. *B*, Quantitation of

secreted GRP in CM collected from LNCaP, LNCaP-zeo, GRP1-1, GRP4-9, and small cell lung carcinoma DMS53 cells. *C*, Androgen-independent growth of GRP1-1 and 4-9 was targeted by inhibitors for GRP and its receptor. All inhibitors reduced the GRP cell growth in CS media with significance. *D*, Boyden chamber migration assay. Conditioned media (CM) were collected from cells by overlaying SF media on sub-confluent plates for 48 hr and the amount to use was normalized by the total protein concentration of each plate. CM from LNCaP-Zeo (ctlCM) or GRP (GRPCM) cells were used as the chemo-attractants. GRP-specific monoclonal antibody 2A11 (1 µg/ml) was introduced as the inhibitor. Bombesin (100 nM) was the positive control. Migration assay was conducted as described in the Materials and Methods. Means of data from at least three independent experiments were plotted and bars represent standard error of the mean.

Figure 2. In vitro (soft agar assay) and in vivo (nude mice) tumorigenesis in androgen-deprived conditions: *A*, Soft agar assay was performed in CS medium as described in Materials and Methods. The experiment has been performed independently three times and the error bars represent standard error of the mean. *B*, Example of orthotopic implanted LNCaP-GRP tumor grown in a castrated nude mouse. Top: whole tumor after 4 months. Bottom: H and E staining showed LNCaP-GRP tumor on left side, mouse prostate stroma in the middle, and normal mouse prostate gland on the right. *C*, IHC staining of GRP (*a* and *b*), AR (*c* and *d*) and PSA (*e* and *f*) in the tumor specimens: Slides on the left (*a*, *c* and *e*) showed most of the normal mouse prostate region; while on the right (*b*, *d* and *f*) showed predominately prostate tumors.

Figure 3. *A*, Soft agar assay of the re-cultured GRP-Pro xenograft: Soft agar assay was performed as described in the Materials and Methods. Treatments include monoclonal antibody to bombesin/GRP, 2A11 (1 µg/ml), anti-androgen bicalutamide (BIC, 5 µM), combination of 2A11 and BIC and synthetic androgen R1881 (1 nM). *B*, Transactivation assay: LNCaP-Zeo,

GRP 4-9 and GRP-Pro cells were plated in CS medium and transfected with the PSA-Luc (630 bp) and pTK-RL. R1881 (1 nM) was added to some wells 24 hours post transfection and dual-luciferase assay was conducted after another 24 hours. Means of triplicate experiments were plotted and bars represent standard error of the mean. *C*, Chromatin immunoprecipitation assay: AR binding to both the enhancer and proximal ARE in the PSA promoter was revealed through PCR analysis using ChIP assay coupled with amplification with primers described in the Materials and Methods. “E”, “P”, and “I” designate for the upstream enhancer region, proximal ARE region, and the intervening region, respectively.

Figure 4. Immunofluorescent staining of AR in LNCaP-Zeo and GRP4-9 cells in response to AZD0530 treatment. AR localization in the nuclei of GRP4-9 cells under androgen-deprived conditions is inhibited by AZD0530. Numbers on the right represent the percentage of cells with AR nuclear localization.

Figure 5. *A*, Effect of AZD0530 on Src/FAK/Etk complex: Phosphorylation status of Src, Lyn and FAK kinases in LNCaP-zeo and GRP subclones was shown in the upper panel. Treatment of all cells with 1  $\mu$ M of AZD0530 for 2 hours diminishes kinase activations in all cells without affect the total protein levels. Association of Src/FAK/Etk complex was illustrated by co-immunoprecipitation with the anti-FAK antibody. Cell lysates from Zeo and GRP cells were immunoprecipitated with anti-FAK antibody and probed for p-Etk, p-FAK, total FAK p-Src and total Src antibodies. Numbers under the untreated samples represent the densitometric quantification for phosphorylation after normalized by the total protein loading. *B*, Knocking down Src with siSrc transfection impaired the androgen-independent growth of GRP4-9 and GRP-Pro cells in CS media. LNCaP-Zeo cells were used as the control for GRP cells and SC (scramble control) was used as the control for siSrc. Western blots validated the siSrc transfection. *C*, Effect of AZD0530 and siSrc transfection on migration: AZD0530 (500 nM) or

knocking down Src kinase with siSrc inhibited GRP4-9 and GRP-Pro cell migration. SC (scramble control) was used as the control for siSrc. The experiment has been performed independently at least three times and the error bars represent standard error of the mean.

Figure 6. In vivo inhibition study in SCID mice: *A*, The representative picture showed primary prostate tumor with lymph node metastasis in an animal from the control group. H&E staining of the lymph node sample validates its prostate cancer origin. *B*, IHC staining of the control and AZD0530 treated tumor samples with anti-p-Src (Y419), anti-p-FAK (Y861) and anti-AR (PG-21) antibodies. *C*, Means of PSA levels in sera, primary tumor weight and metastasis incidents were plotted between the control and AZD0530 treatment groups.

## REFERENCES

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA: a cancer journal for clinicians* 2007;57:43-66.
2. Culig Z, Steiner H, Bartsch G, Hobisch A. Interleukin-6 regulation of prostate cancer cell growth. *Journal of cellular biochemistry* 2005;95:497-505.
3. Lee LF, Guan J, Qiu Y, Kung HJ. Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. *Mol Cell Biol* 2001;21:8385-97.
4. Lee LF, Louie MC, Desai SJ, *et al.* Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. *Oncogene* 2004;23:2197-205.
5. Burchardt T, Burchardt M, Chen M.W., Cao Y., de la Taille A., Shabsigh A., Hayek O., Dorai T., Buttyan R. Transdifferentiation of prostate cancer cells to a neuroendocrine cell phenotype in vitro and in vivo. *J Urol* 1999;162:1800-5.
6. Cox ME, Deeb PD, Lakhani S, Parsons SJ. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. *Cancer Research* 1999;59:3821-30.
7. Vashchenko N, Abrahamsson PA. Neuroendocrine differentiation in prostate cancer: implications for new treatment modalities. *Eur Urol* 2005;47:147-55.
8. Cuttitta F, Carney DN, Mulshine J, *et al.* Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature* 1985;316:823-6.
9. Nagata A, Ito M, Iwata N, *et al.* G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa in vivo. *Proc Natl Acad Sci U S A* 1996;93:11825-30.
10. Abrahamsson PA. Neuroendocrine cells in tumour growth of the prostate. *Endocr Relat Cancer* 1999;6:503-19.
11. Rozengurt E. Neuropeptides as growth factors for normal and cancerous cells. *Trends Endocrinol Metab* 2002;13:128-34.
12. Kim S, Hu W, Kelly DR, Hellmich MR, Evers BM, Chung DH. Gastrin-releasing peptide is a growth factor for human neuroblastomas. *Ann Surg* 2002;235:621-9; discussion 9-30.

13. Guha S, Lunn JA, Santiskulvong C, Rozengurt E. Neurotensin stimulates protein kinase C-dependent mitogenic signaling in human pancreatic carcinoma cell line PANC-1. *Cancer Res* 2003;63:2379-87.
14. Aprikian AG, Han K, Guy L, Landry F, Begin LR, Chevalier S. Neuroendocrine differentiation and the bombesin/gastrin-releasing peptide family of neuropeptides in the progression of human prostate cancer. *Prostate Supplement* 1998;8:52-61.
15. Festuccia C, Guerra F, S DA, Giunciuglio D, Albin A, Bologna M. In vitro regulation of pericellular proteolysis in prostatic tumor cells treated with bombesin. *International Journal of Cancer* 1998;75:418-31.
16. Wu JT, M.E. Astill, G.H. Liu, and R.A. Stephenson. Serum chromogranin A: early detection of hormonal resistance in prostate cancer patients. *J Clin Lab Anal* 1998;12:20-5.
17. Bartholdi MF, Wu JM, Pu H, Troncoso P, Eden PA, Feldman RI. In situ hybridization for gastrin-releasing peptide receptor (GRP receptor) expression in prostatic carcinoma. *Int J Cancer* 1998;79:82-90.
18. Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. *Cancer Res* 1999;59:1152-9.
19. Luttrell LM, Daaka Y, Lefkowitz RJ. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 1999;11:177-83.
20. Aprikian AG, Han K, Chevalier S, Bazinet M, Viallet J. Bombesin specifically induces intracellular calcium mobilization via gastrin-releasing peptide receptors in human prostate cancer cells. *Journal of Molecular Endocrinology* 1996;16:297-306.
21. Han K, J. Viallet, S. Chevalier, W. Zheng, M. Bazinet, and A.G. Aprikian. Characterization of intracellular calcium mobilization by bombesin-related neuropeptides in PC-3 human prostate cancer cells. *Prostate* 1997;31:53-60.
22. Siegfried JM, Guentert PJ, Gaither AL. Effects of bombesin and gastrin-releasing peptide on human bronchial epithelial cells from a series of donors: individual variation and modulation by bombesin analogs. *Anat Rec* 1993;236:241-7.
23. Cai RZ, Reile H, Armatas P, Schally AV. Potent bombesin antagonists with C-terminal Leu-psi(CH<sub>2</sub>-N)-Tac-NH<sub>2</sub> or its derivatives. *Proc Natl Acad Sci U S A* 1994;91:12664-8.
24. Sausville EA, Lebacqz-Verheyden AM, Spindel ER, Cuttitta F, Gazdar AF, Battey JF. Expression of the gastrin-releasing peptide gene in human small cell lung cancer. Evidence for alternative processing resulting in three distinct mRNAs. *J Biol Chem* 1986;261:2451-7.
25. Desai SJ, Ma AH, Tepper CG, Chen HW, Kung HJ. Inappropriate activation of the androgen receptor by nonsteroids: involvement of the Src kinase pathway and its therapeutic implications. *Cancer Res* 2006;66:10449-59.
26. Louie MC, Yang HQ, Ma AH, *et al.* Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. *Proc Natl Acad Sci U S A* 2003;100:2226-30.
27. Chang YM, Bai L, Liu S, Yang JC, Kung HJ, Evans CP. Src family kinase oncogenic potential and pathways in prostate cancer as revealed by AZD0530. *Oncogene* 2008.
28. Guo Z, Dai B, Jiang T, *et al.* Regulation of androgen receptor activity by tyrosine phosphorylation. *Cancer Cell* 2006;10:309-19.
29. Dai B, Kim O, Xie Y, *et al.* Tyrosine kinase Etk/BMX is up-regulated in human prostate cancer and its overexpression induces prostate intraepithelial neoplasia in mouse. *Cancer Res* 2006;66:8058-64.
30. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nature reviews* 2005;6:56-68.
31. Chen R, Kim O, Li M, *et al.* Regulation of the PH-domain-containing tyrosine kinase Etk by focal adhesion kinase through the FERM domain. *Nat Cell Biol* 2001;3:439-44.
32. Culig Z, Hobisch A, Cronauer MV, *et al.* Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* 1994;54:5474-8.
33. Jin RJ, Wang Y, Masumori N, *et al.* NE-10 neuroendocrine cancer promotes the LNCaP xenograft growth in castrated mice. *Cancer Res* 2004;64:5489-95.
34. Thomas SM, Grandis JR, Wentzel AL, Gooding WE, Lui VW, Siegfried JM. Gastrin-releasing peptide receptor mediates activation of the epidermal growth factor receptor in lung cancer cells. *Neoplasia* 2005;7:426-31.
35. Paronetto MP, Farini D, Sammarco I, *et al.* Expression of a truncated form of the c-Kit tyrosine kinase receptor and activation of Src kinase in human prostatic cancer. *Am J Pathol* 2004;164:1243-51.
36. Gioeli D, Ficarro SB, Kwiek JJ, *et al.* Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J Biol Chem* 2002;277:29304-14.

37. Gong J, Zhu J, Goodman OB, Jr., *et al.* Activation of p300 histone acetyltransferase activity and acetylation of the androgen receptor by bombesin in prostate cancer cells. *Oncogene* 2006;25:2011-21.
38. Zheng R, Iwase A, Shen R, *et al.* Neuropeptide-stimulated cell migration in prostate cancer cells is mediated by RhoA kinase signaling and inhibited by neutral endopeptidase. *Oncogene* 2006;25:5942-52.
39. Mao J, Xie W, Yuan H, Simon MI, Mano H, Wu D. Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Galpha12/13. *The EMBO journal* 1998;17:5638-46.
40. Tsai YT, Su YH, Fang SS, *et al.* Etk, a Btk family tyrosine kinase, mediates cellular transformation by linking Src to STAT3 activation. *Mol Cell Biol* 2000;20:2043-54.
41. Aprikian AG, Tremblay L, Han K, Chevalier S. Bombesin stimulates the motility of human prostate-carcinoma cells through tyrosine phosphorylation of focal adhesion kinase and of integrin-associated proteins. *Int J Cancer* 1997;72:498-504.
42. Jiang X, Borgesi RA, McKnight NC, Kaur R, Carpenter CL, Balk SP. Activation of Nonreceptor Tyrosine Kinase Bmx/Etk Mediated by Phosphoinositide 3-Kinase, Epidermal Growth Factor Receptor, and ErbB3 in Prostate Cancer Cells. *J Biol Chem* 2007;282:32689-98.
43. Zhou J, Chen J, Mokotoff M, Ball ED. Targeting gastrin-releasing peptide receptors for cancer treatment. *Anticancer Drugs* 2004;15:921-7.
44. Druker BJ, Talpaz M, Resta DJ, *et al.* Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344:1031-7.
45. Verweij J, van Oosterom A, Blay JY, *et al.* Imatinib mesylate (STI-571 Glivec, Gleevec) is an active agent for gastrointestinal stromal tumours, but does not yield responses in other soft-tissue sarcomas that are unselected for a molecular target. Results from an EORTC Soft Tissue and Bone Sarcoma Group phase II study. *Eur J Cancer* 2003;39:2006-11.
46. Slamon DJ, Leyland-Jones B, Shak S, *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001;344:783-92.
47. Hiscox S, Jordan NJ, Morgan L, Green TP, Nicholson RI. Src kinase promotes adhesion-independent activation of FAK and enhances cellular migration in tamoxifen-resistant breast cancer cells. *Clinical & experimental metastasis* 2007;24:157-67.
48. Gautschi O, Tepper CG, Purnell PR, *et al.* Regulation of Id1 expression by SRC: implications for targeting of the bone morphogenetic protein pathway in cancer. *Cancer Res* 2008;68:2250-8.
49. Nam S, Kim D, Cheng JQ, *et al.* Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. *Cancer Res* 2005;65:9185-9.
50. Park SI, Zhang J, Phillips KA, *et al.* Targeting SRC family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model. *Cancer Res* 2008;68:3323-33.